Department of Biotechnology and Chemical Technology

Pretreatment categories, process alternatives and material characteristics in enzymatic hydrolysis of lignocellulose

Ville Pihlajaniemi

Aalto University

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Ville Pihlajaniemi

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Abstract

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Abstract

Fractionation of lignocellulose materials to sugars is a major strategy for the production of renewable fuels and chemicals. This study compares the potential of two major pretreatment categories, hydrothermal treatment and delignification, and contributes to scientific understanding of the phenomena behind enzymatic hydrolysability of wheat straw. Delignification was found to allow higher sugar yields. Since enzyme consumption is a key cost of the fractionation process, the optimal yield target depends on enzyme price. To allow yield optimization, a novel empirical model was developed for the process sugar yield as a function of enzyme consumption and hydrolysis time. The usability of the model was demonstrated by comparing the feasibility of different process alternatives for fractionation.

The changes in the material properties of lignocellulose by pretreatments were correlated to cellulose hydrolysability, and for the first time, the importance of the different properties was determined statistically. In the order of importance, the hydrolysis yield depended on cellulose surface area, pore accessibility, lignin content, lignin surface chemistry, cellulose crystallinity and hemicellulose content. During enzymatic hydrolysis, the surface area of cellulose correlated linearly with the total cellulose content, but contrary to expectations, hydrolysis did not reveal fresh lignin surfaces. Different rate constraining mechanisms were incorporated in a Michaelis-Menten type kinetic model, and it was found that permanent hydrolysis-dependent enzyme inactivation should be included with the previously well-established effects of product inhibition and reduction of hydrolysability.

For improving fractionation processes, different technological solutions were studied. A flow through process was found to improve fractionation by delignification, but no additional improvement was achieved by counter-current operation. By studying and simulating the packing density and flow properties of a packed straw bed, a flow-through process was found to be possible without clogging the straw bed by compaction. The height of an industrial scale column is restricted by the applicable flow rate. With the simulation model, it was possible to determine the maximum volumetric throughput as a function of column height.

Recycling of the solid residue during enzymatic hydrolysis was found to be inefficient for enzyme recycling, but efficient for product removal, with similar benefits as sequential hydrolysis. Both processes significantly improved the volumetric productivity of hydrolysis by increasing the solids concentration without reducing yield. Alternatively, this benefit could be redirected into increasing the yield by maintaining reaction volume with additional water, leading to dilution of the hydrolysis conditions.

Keywords Lignocellulose hydrolysis, cellulase, pretreatment, wheat straw, yield optimization

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Tiivistelmä

Lignoselluloosamateriaalien fraktiointia ja pilkkomista sokereiksi on tutkittu laajasti uusiutuvien polttoaineiden ja kemikaalien tuottamiseksi. Tässä tutkimuksessa vertailtiin kahden tärkeimmän esikäsittelykategorian, hydrotermisen käsittelyn ja delignifioinnin, vaikutusta vehnän oljesta entsyymihydrolyysillä saatavaan sokerisaantoon ja sen taustalla oleviin ilmiöihin. Suuremmat sokerisaannot saavutettiin delignifioinnilla. Koska entsyymikulutus on yksi prosessin avainkustannuksista, saantotavoite tulisi optimoida entsyymin hinnan mukaan. Tätä varten kehitettiin uusi empiirinen malli sokerisaannolle entsyymikulutuksen ja reaktioajan funktiona, ja mallin käyttöä havainnollistettiin suoraviivaisella kannattavuusanalyysillä eri fraktiointiprosesseille.

Esikäsittelyjen vaikutuksia lignoselluloosan materiaaliominaisuuksiin vertailtiin hydrolyysisaantoihin ja eri ominaisuudet asetettiin ensimmäistä kertaa tilastollisesti tärkeysjärjestykseen seuraavasti: selluloosan pinta-ala, huokosrakenne, ligniinin määrä, ligniinin pintakemia, selluloosan kiteisyys ja hemiselluloosan määrä. Selluloosan pinta-ala pieneni lineaarisesti selluloosamäärän kanssa, kun taas ligniinin pinta-ala pysyi lähes muuttumattomana, vaikka hydrolyysin odotettiin paljastavan tuoreita ligniinipintoja. Erilaisia reaktionopeutta laskevia tekijöitä arvioitiin yhdistämällä niitä Michaelis-Menten –tyyppiseen kinetiikkamalliin. Havaittiin että aiemmissa malleissa huomioitujen lopputuoteinhibition ja hydrolysoituvuuden laskun lisäksi malliin on perusteltua lisätä hydrolyysiasteesta riippuva peruuttamaton inhibitiotekijä.

Fraktioinnin tehostamiseksi tutkittiin vaihtoehtoisia prosessiratkaisuja. Läpivirtausprosessin havaittiin parantavan sokerisaantoja delignifioinnissa, mutta vastavirtaprosessi ei tuonut lisäparannusta suoraan läpivirtaukseen verrattuna. Olkipedin pakkaustiheyttä ja virtausominaisuuksia määritettiin ja simuloinnin avulla läpivirtausprosessi havaittiin käyttökelpoiseksi suuressa mittakaavassa. Oljen kokoonpuristuminen rajoitti käytettävää virtausnopeutta, mikä taas vaikutti kolonnin kapasiteettiin ja korkeuteen. Kolonnin kapasiteetti määritettiin korkeuden funktiona simuloinnin avulla.

Kiintoaineen kierrätys entsyymihydrolyysissä ei toiminut entsyymien kierrätysmenetelmänä, mutta oli sen sijaan tehokas tapa poistaa tuotetta, yhtälailla kuin moninkertainen hydrolyysi. Kumpikin tehosti merkittävästi volumetrista tuottavuutta mahdollistamalla korkeamman kuiva-ainepitoisuuden ilman saannon laskua. Vaihtoehtoisesti hyöty kyettiin ohjaamaan hydrolyysisaannon kasvuksi vakioimalla reaktiotilavuus, jolloin veden lisäys johti kokonaisprosessiolosuhteiden laimenemiseen.

Avainsanat Lignoselluloosan hydrolyysi, sellulaasi, esikäsittely, vehnän olki, optimointi

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Preface

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Contents

List of publications

I Pihlajaniemi, V., Sipponen, M.H., Pastinen, O., Lehtomäki, I., Laakso, S. Yield optimization and rational function modeling of enzymatic hydrolysis of wheat straw pretreated by NaOH-delignification, autohydrolysis and their combination. *Green Chem,* **17,** 1683-1691 (2015).

II Pihlajaniemi, V., Sipponen, M.H., Liimatainen, H., Sirviö, J.A., Nyyssölä, A., Laakso, S. Weighing the factors behind hydrolysability of lignocellulose materials. *Green Chem.* ***18,*** 1295–1305 (2016).

III Pihlajaniemi, V., Sipponen, M.H., Kallioinen, A., Nyyssölä, A., Laakso, S. Rate constraining changes in surface properties, porosity and hydrolysis kinetics of lignocellulose in the course of enzymatic saccharification. *Biotechnol. Biofuels* **9**:18, 1–12 (2016).

IV Pihlajaniemi V., Sipponen, M.H., Pastinen O., Nyyssölä, A., Laakso, S. The effect of direct and counter-current flow-through delignification on enzymatic hydrolysis of wheat straw and flow limits due to compressibility. *Biotechnol. Bioeng.* In press (2016). DOI: 10.1002/bit.26030.

V Pihlajaniemi, V., Sipponen, S., Sipponen, M.H, Pastinen, O., Laakso, S. Enzymatic saccharification of pretreated wheat straw: Comparison of solids-recycling, sequential hydrolysis and batch hydrolysis. *Bioresour. Technol.* **153,** 15-22 (2014).

Author contributions

I This article was based on several years of pretreatment and hydrolysis research performed by all of the authors. Based on this experience, VP designed the study with input from MS and SL, performed the pretreatment and hydrolysis reactions and titrations, processed the results, produced the empirical model and wrote the manuscript. MHS performed the compositional analysis of pretreated straw samples and HPSEC analysis of lignin and modified the chapter "Molar mass of dissolved lignin". OP performed the organic acid analysis by HPLC and provided expertise for sugar analysis. The manuscript was critically reviewed by MHS, SL and others.

II This study was designed by VP with input from MHS and SL as a continuation to **I**. VP performed the Congo Red adsorption, thermoporometry and FTIR analysis, processed the results, performed the principal component analysis and wrote the manuscript. MHS performed the Azure B adsorption and HL and JAS performed WAXD-analysis of cellulose crystallinity. The manuscript was critically reviewed by AN, MHS, SL and others.

III VP designed the study with significant contribution from MHS. VP performed the hydrolysis reactions, cellulose surface analysis, thermoporometry and modelling, processed the results and wrote the manuscript. MHS produced the delignified straw and performed the lignin surface analysis, AK performed the phenolic analysis and the product inhibition experiment and Soila Saavala performed the residual activity determination. The manuscript was critically reviewed by AN, MHS, SL and AK.

IV This study was designed by VP, who performed the flow-through reactions and hydrolysis, titrations, dynamic pressure drop determinations and simulations, and wrote the manuscript. MHS performed lignin determination by HPSEC. Large batch delignification and straw compressibility tests were performed by Soila Saavala. The manuscript was critically reviewed by AN, MHS and SL.

V This study was designed by VP, who performed the hydrolysis reactions with assistance from SS, processed the results and wrote the manuscript. MHS produced the delignified straw and OP provided expertise for sugar analysis. The manuscript was critically reviewed by SL, MHS and OP.

List of abbreviations

AFEX	Ammonia fiber explosion
AH	Autohydrolysis
ARP	Ammonia recycle percolation
ATR-FTIR	Attenuated total reflection Fourier-transform infrared spectroscopy
BET	Brunauer-Emmett-Teller
CBH	Cellobiohydrolase
CBM	Cellulose binding module
CrI	Crystallinity index
DM	Dry matter
FPU	Filter paper unit
GAE	Gallic acid equivalent
GHG	Greenhouse gas
HPLC	High performance liquid chromatography
HPSEC	High performance size-exclusion chromatography
LPMO	Lytic polysaccharide monooxygenase
MM	Michaelis-Menten
NREL	National Renewable Energy Laboratory (USA)
PEG	Polyethylene glycol
SD	Standard deviation
SSA	Specific surface area
SSF	Simultaneous saccharification and fermentation
tpDSC	Thermoporometry by differential scanning calorimetry
WAXD	Wide angle X-ray diffractometry

1 Introduction

Along with ever growing global oil demand, concerns about depletion of fossil fuel reserves arise periodically.[1–3] The depletion of inexpensive fossil oil sources has been a recurring concern, which has so far mainly led to the discovery of new sources and technologies for replenishing the fossil fuel supply,[4] but also accelerated the search for alternative fuels. Continuous debate about greenhouse gas (GHG) emissions and climate change creates demand for sustainable energy sources, and the development of renewable alternatives for fuel and chemical production is encouraged with legislative mandates and emission trading systems. Biofuels offer a sustainable alternative to fossil fuels. They also carry a promise of a sovereign energy source for those with no access to the unevenly distributed fossil energy reserves. Given the wide range of motivations for decreasing dependence on fossil fuels, biofuels are a subject of strategic research for many countries and institutions.

Cellulose is the most abundant organic material on earth, and together with hemicellulose and lignin, it comprises lignocellulose, the main structural material of plants. Different lignocellulose materials are available as waste and residues from agriculture and forestry, and these materials, including straw, corn stover, sugarcane bagasse and waste wood, as well as dedicated energy crops such as switchgrass or mischantus, could provide an immense source of renewable feedstocks for the production of biofuels and chemicals.[5–7] The evolution of lignocellulosic materials, however, has led to a structure that is very recalcitrant towards microbial, enzymatic and chemical breakdown. Tremendous research efforts have therefore been undertaken to develop different approaches for their utilization.

The classical biotechnological conversion of lignocellulose into fuels and chemicals consists of pretreatment, enzymatic hydrolysis and fermentation (Figure 1). The pretreatment breaks down the physicochemical structure of the material, facilitating subsequent enzymatic hydrolysis. The pretreatments can be divided into two major categories: acid-catalyzed breakdown of hemicellulose, and delignification by alkaline treatment, organic solvents or oxidative chemicals.[8,9] The cellulose and hemicellulose in the pretreated material are hydrolysed into monomeric sugars by a range of cellulase and hemicellulase activities, and converted to biofuel components or chemicals by fermentation.[10,11]

High enzyme consumption and insufficient sugar yields are still major barriers for the economical viability of the process.[12] Cellulose is a solid, crystalline material, and compounded with lignin and hemicellulose, it forms a heterogenous material with limited enzyme accessibility to cellulose surfaces.[13]

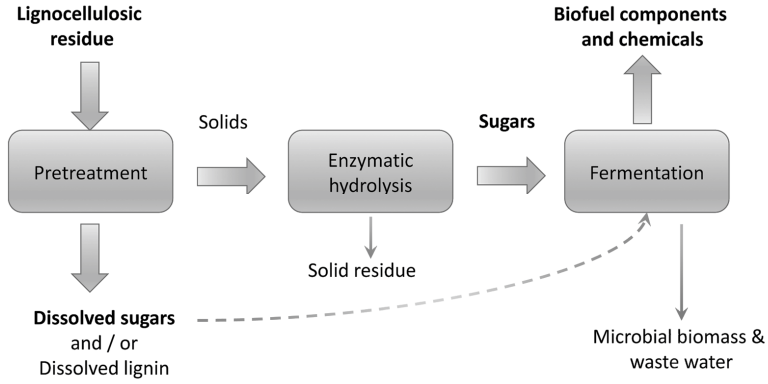

Figure 1. *Fermentation route of the production of biofuels and chemicals.*

The presence of lignin is a major hindrance, not only as a barrier, but also as an inhibitor of cellulases.[14,15] Additionally, cellulolytic enzymes suffer from product inhibition by sugars, and the long reaction times under thermally and mechanically harsh conditions may lead to denaturation of the enzymes.[16,17] Although many hydrolysis-constraining effects have been described, their relative importance is still under debate and conclusion has not been reached concerning the hydrolysis kinetics of lignocellulose.[18–20] Apart from the development of enzymes themselves, approaches for directly reducing enzyme consumption include enzyme recycyling[21], applying hydrolysis additives[22], optimization of the enzyme cocktail[23] and product removal to decrease product inhibition[24].

Transportation fuels are relatively low-value products, which leaves little room for process complexity and chemical consumption. Maximizing process yields while minimizing the number of unit operations and operational costs is a challenge and a guideline for the evaluation of process alternatives, and complicated approaches can often be quickly ruled out from the list of near future solutions.[12,25,26] However, given the large number of material properties and process parameters affecting the outcome, the comparison of the potential of different processes is not straightforward.

1.1 Eventual Transition towards renewables

Growing ecological awareness, worries about depletion of fossil oil reserves and hopes to reduce dependence on imported oil have driven the research of biofuels for decades. The Renewable Energy Directive of the EU targets at 20% renewable energy sources and 10% renewable transportation fuels by 2020, while the national renewable targets of Finland have been set at 38% of energy

and 20% of transportation fuels.[27,28] The "Renewable Fuel Standard" of USA aims at approximately 7% renewable fuels, including 3% of cellulosic fuels, of the total fuel consumption.[29] Additionally, periodic concerns about "Peak oil" have envisioned dramatic global consequences of a sudden collapse of cheap oil supplies.[1,2]

The majority of the current biofuel production consists of ethanol produced from corn (USA) and sugarcane (Brazil), and biodiesel from plant oils (Europe). However, these biofuels compete with food production, and this controversy is sought to be averted by a transition to lignocellulosic biofuels.[30] An alternative approach has been the production of biodiesel from oil-containing algae. The algae fuels have, however, shown severe lag in reaching a competitive price level, with a low productivity and difficulties in harvesting and extraction.[27,31]

The development of lignocellulosic biofuels has been slow but steady for decades. However, since 2007 the high and volatile oil prices have accelerated their development. It has been stated that the highest potential for the improvement of the production of lignocellulosic biofuels currently lies in the commercialization learning curve, rather than studying the process at lab scale[25,26,32]. Accordingly, the recent wave of biofuel research and development has seen demonstration and pre-commercial plant investments of unprecedented scale, including those of POET and DuPont in the USA, Granbio and Raizen in Brazil, Iogen (Canada), SunOpta (China), Beta-Renewables (Italy) and Inbicon (Denmark).[25,27,33] Nevertheless, enzyme consumption remains the key cost of the process, and major research efforts on the pretreatment and hydrolysis processes and enzymes themselves have been made in order to reach economic viability.[12]

Besides legislative mandates, the incentive to invest in lignocellulosic biofuels has relied on projections of a growing oil price, which turned to the contrary in 2014.[34] The high oil price also led to the development of the recovery technology of shale oil, thus unlocking new reserves of unconventional fossil fuels, particularly in the USA.[35] Coinciding with slowing economic growth in China and Europe, the oil price has fallen to 44% of its 10-year average by January 2016 (Index Mundi[*]). Because of the economic situation and the expected reduction in OPEC influence on oil price, a near future return to the previous high levels does not seem likely. Additionally, the price of sugar, a benchmark for lignocellulose saccharification, has declined 40–50% in the last five years. These developments have cast a shadow over the development of lignocellulosic fuels.

Nevertheless, the "shale oil boom" serves as an example of a transition to an alternative technology, if the price of oil reaches sufficiently high levels. As the conventional oil reserves will eventually decline, this transition will ultimately lead towards renewable sources. The availability of alternatives therefore

[*] http://www.indexmundi.com/commodities/?commodity=crude-oil&currency=eur, Feb 2016.

implies that, instead of a disastrous oil depletion crisis, a much smoother route can be expected.[3] This highlights the strategic nature of the development of lignocellulosic biofuels. The current oversupply of oil will eventually be balanced and the global incentive towards renewable fuels is strong, as demonstrated by the Paris Climate change agreement in 2015. The commercial potential of lignocellulosic biofuels has already been demonstrated and the first round on the commercialization learning curve has been completed. The technology now awaits a favorable turn in economic conditions to trigger a second round.

1.2 Biofuels and chemicals from lignocellulosic sugars

The primary potential products from lignocellulosic sugars are large scale, low value products, such as fuels and bulk chemicals for two reasons. First, the controversy of using starch-based sugars is only an issue at high raw-material input, whereas for lower volume fine chemicals, starch based sugars are better justified, particularly if high purity feedstocks are a priority. Second, the economies of scale are needed to improve the feasibility of the lignocellulose saccharification process.[26] However, the limiting factor for the scale of lignocellulosic processing is the availability and logistics of the raw materials, the sources of which are widely distributed. The optimal plant size therefore depends on location, with estimates in the USA ranging between 2000 and 14000 tons per day, although the capacity of the currently existing lignocellulosic ethanol plants is considerably lower.[29]

While ethanol is the predominant lignocellulosic biofuel component, biobutanol is a promising alternative, with a higher energy density (36 vs. 27 MJ kg^{-1}) and is less corrosive to engines compared to ethanol.[10] Butanol is, however, more toxic to the production organism, leading to a low volumetric productivity and concentration. A general challenge of alcohol production is the inefficient conversion of the hemicellulosic pentose (C5) sugars such as xylose by alcohol-producing yeasts. Efficiency has been improved with genetic engineering and separate pentose fermentation, but an efficient process is still seen as a future scenario.[36,37] A third option is microbial oil, which can be used as a feedstock for renewable diesel in a similar way as plant oils. It has the highest energy density (41 MJ kg^{-1}) and oil-producing fungi are readily able to use C5-sugars as a carbon source. However, the cultivation is aerobic and the oil is intracellular, leading to additional costs of aeration and extraction compared to alcohols.[11,38] Aside from biofuels, many other large-scale biochemicals can be obtained from lignocellulosic sugars, including dicarboxylic and hydroxy acids, which are precursors of different chemicals and polymers.[39] Regardless of the main

product, the valorization of the process side streams, particularly lignin, is essential for the process economy.[40]

Given the long reaction times in lignocellulose hydrolysis and fermentation, the volumetric productivity of the process can be improved by combining these operations into a process called simultaneous saccharification and fermentation (SSF).[36,41] SSF is also beneficial for hydrolysis, since the produced sugars are consumed simultaneously as they are released, which reduces product inhibition of cellulases.

The biotechnological sugar route competes with thermochemical conversion of lignocellulose, particularly pyrolysis and gasification. Pyrolysis is the degradation of biomass at high temperatures, producing a tar-like low quality "pyrolysis-oil", which is combustible, but requires further refining to valuable products. Gasification produces syngas, which can be transformed into different liquid fuels and chemicals through a versatile but expensive process called the Fischer-Tropsch synthesis.[42,43]

1.3 Lignocellulose materials and enzymes

A common name for the major material of plants is lignocellulose, which is composed of an intertwining network of cellulose, hemicellulose and lignin. Located in the plant cell walls, it forms the durable structure of wood, stems and straw. The cell wall is a layered network of cellulose fibrils, bound together by non-covalent interactions with hemicellulose. Hemicellulose is covalently linked to lignin, which fills the spaces and provides rigidity, insulation and protection

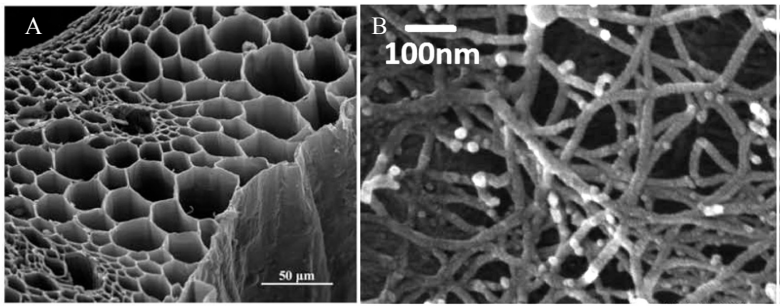

Figure 2. *A. Scanning electron microscopy images of A) a wheat straw cross section by Kristensen et al.,[44] B. the fibril network of a delignified kraft pulp fibre, (modified from Duchesne & Daniel[45] by Peciulyte et al.[46])*[†]

[†] Reprinted with permission from Nordic Pulp and Paper Research Journal.

against microbial attack. The cell wall is porous, with the pore sizes at nanometer scale, whereas the lumen of the cell is typically of micrometer scale (Figure 2A).[5,47–49]

Cellulose is a linear polysaccharide consisting of glucose (β-D-glucanopyranose) units, linked by 1,4-glycosidic bonds. Neighboring glucanopyranosyl units within and between individual cellulose molecules form hydrogen bonds with each other to form a crystalline elementary fibril structure.[48–50] These fibrils are aggregated together, with the help of associated hemicellulose, to form networks of larger fibrils (Figure 2A & Figure 3A).[45,51] Hemicelluloses are branched heteropolysaccharides with highly diverse composition with the major monosaccharides xylose, arabinose, galactose, mannose and glucose. Arabinoxylan is the major hemicellulose in grasses, where arabinose substituents

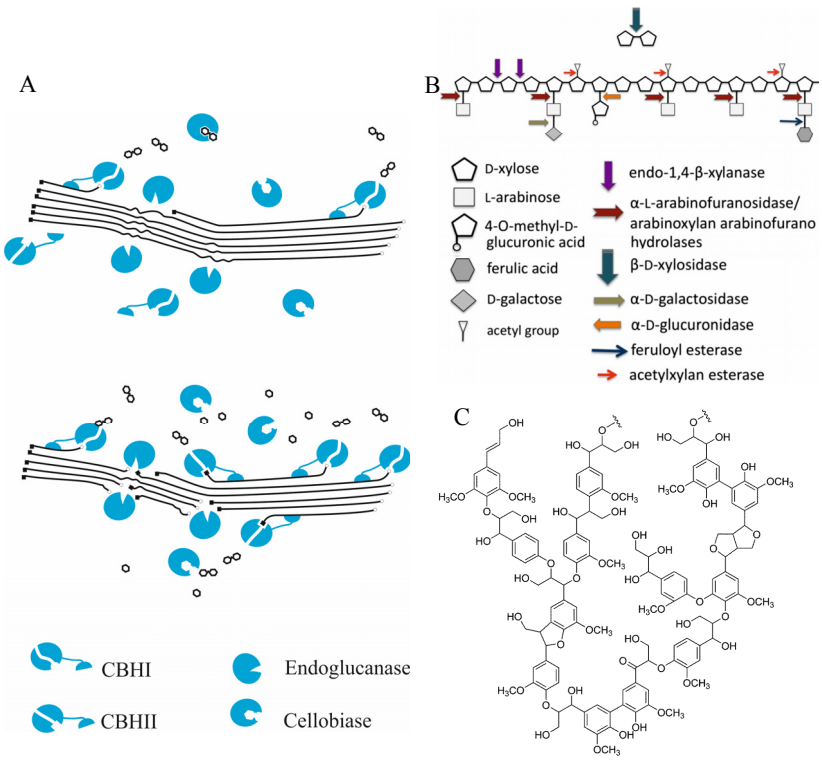

Figure 3. *Structure and enzymatic hydrolysis of A) cellulose and B) hemicellulose (adapted from De Souza et al.[52]). C) The structure of lignin.*[53‡]

‡ Reprinted with permission from the Royal Society of Chemistry

often work as branching points for the xylan backbone and attachment sites to galactopyranosyl units and hydroxycinnamic acid esters (Figure 3B). The latter serve as covalent bridges to other hemicellulose molecules and lignin.[54] Additionally, various amounts of 4-O-methylglucuronic acid and acetyl groups are attached to the xylan backbone. In contrast to grasses, the major hemicellulose components of soft- and hardwoods are galactoglucomannan and glucuronoxylan, respectively.[55]

Lignin is an amorphous, heterogenous resin of randomly polymerized phenolic units, namely *p*-coumaryl, sinapyl and coniferyl alcohols, known as the monolignols. They are irregularly linked with a variety of ether and carbon-carbon bonds leading to branching and cyclic structures.[56] Lignin is hydrophobic, but it also contains free phenolic hydroxyl groups, which are slightly acidic and thus deprotonated under alkaline conditions. This facilitates the dissolution of lignin in alkaline solutions after depolymerization reactions that occur during pulping as well as alkaline pretreatments for hydrolysis. Straw lignins are particularly alkali soluble, which is explained by the abundance of hydrolysable ester bridges linking lignin to hemicellulose. They also differ from wood lignin in their monolignol composition.[54] Although some peroxidases and laccases catalyze radical reactions that can depolymerize lignin[57], enzymatic lignin degradation has so far had little practical significance within the concept of saccharification.

Synergistic action of multiple cellulose and hemicellulose degrading enzymes is required for the saccharification of lignocellulose substrates (Figure 3A). Cellobiohydrolase I (CBHI) and II (CBHII) are exocellulases, which cleave cellobiose units from the cellulose molecule at the reducing end and the non-reducing end, respectively. Endocellulases (β-1,4-endoglucanases) cleave intrachain glycosidic bonds at amorphous regions of cellulose, creating new chain ends for the CBH's. Cellobiases (β-glucosidases) cleave the released cellobiose into glucose.[47,58–60] Typical cellulase enzymes consist of two domains, a cellulose binding module (CBM) and a catalytic domain. Cellobiohydrolases attach to the surface of crystalline cellulose by the CBM and a loose end of a cellulose molecule is bound to the tunnel-like active center. The enzyme then advances along the fibril, detaching the cellulose strand from the crystal and hydrolyzing it in a processive manner.[58,61] Oxidoreductive cellulases and proteins that induce swelling of cellulose can also contribute synergistically to cellulose depolymerization.[62,63]

In comparison with cellulose, hemicelluloses are more heterogenous substrates and therefore require a higher number of different enzyme activities for complete hydrolysis (Figure 3B). The major enzyme for the cleavage of the main chain is xylanase, since xylan is the major component of hemicellulose in many plants, including grasses. Other activities include arabinases, galactosidases, glucuronidases and mannanases for the main chain and its substituents, acetyl

esterases for the acetyl groups and feruloyl esterases to break the ferulic acid cross-links to lignin.[52,64–66]

1.4 Pretreatment of lignocellulose materials

A large number of pretreatment methods have been proposed in order to facilitate enzymatic hydrolysis of lignocellulose. Most of the processes fall into two categories: hydrothermal (acidic) treatments and delignification. Many combinatory and exploratory methods have also been suggested.

1.4.1 Hydrothermal and dilute acid pretreatment

Hydrothermal and dilute acid treatments are the most common pretreatment processes in the current demonstration plants for lignocellulosic biofuels.[25,27,67] Hydrothermal pretreatment, also known as autohydrolysis (AH), is a simple treatment of lignocellulose with water at high temperature and pressure, typically between 180 and 220 ºC for 5 to 20 min.[23,68] These conditions lead to the cleavage of most of the acetyl groups of hemicellulose as acetic acid, which then catalyses the hydrolysis of hemicellulose. The extent of hemicellulose dissolution depends on the combination of temperature and time, which is described by the severity parameter $\mathrm{Log}(R_0)$.[69] Excessively severe conditions lead to dehydration of the dissolved sugars into furans and further into organic acids. The majority of cellulose and lignin remain in the pretreated solids (Figure 4). Lignin, however, undergoes physical and chemical changes, partially translocating into coalescent droplets at the material surface, and forming condensation products with carbohydrates, known as pseudo-lignin.[70,71]Typically up to 60–70% of the hemicellulosic sugars are recovered in the autohydrolysis liquid, mostly in oligomeric form.[23,68,72] The addition of dilute acid further improves hemicellulose dissolution and its hydrolysis to monomeric sugars at lower temperatures compared to autohydrolysis. This leads to potentially lower degradation losses, with the cost of the chemical addition and neutralization.[73–77]

At excess enzyme dosages, cellulose hydrolysis yields from AH-straw up to 90–96% have been reported,[72,73] as well as ~84% of the theoretical conversion to ethanol.[23] On the other hand, cellulose saccharification at "realistic" enzyme dosages and reaction conditions (below 10 FPU g^{-1} cellulose, consistency above 10% DM) have only led to 60–70% yields from the original glucan.[12,78] Since the optimum severity for dissolved hemicellulose recovery is lower than that for enzymatic hydrolysability of cellulose, a two-stage autohydrolysis has been proposed[79] with the recovery of dissolved hemicellulose being up to 83%.

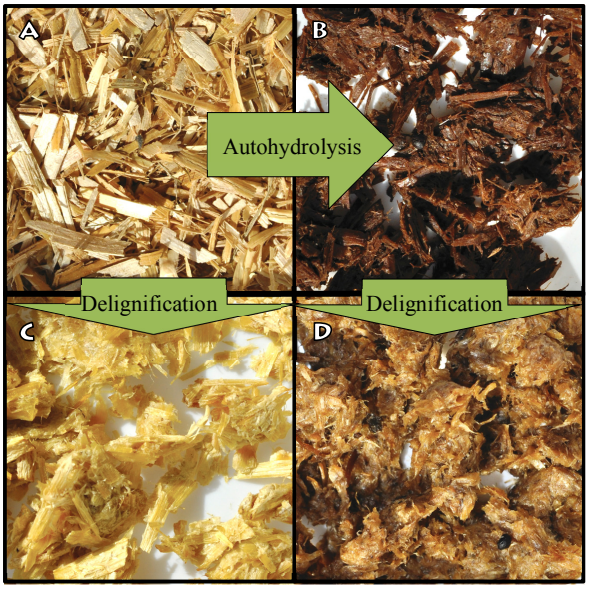

Figure 4. *Wheat straw before and after different pretreatments. A) Native straw, B) autohydrolysed straw, C) delignified straw, D) double-treated straw after autohydrolysis and subsequent delignification.*

However, this approach was later discouraged due to the expenses of the additional unit operations.[25]

The recovered pentose sugars are mixed with acetic and formic acid, phenolics and furfural, which are inhibitory to microbial growth. This has called for studies of detoxification of the hydrolysate,[80] but it has also been used for asepticity control in fermentation.[25] Industrial scale autohydrolysis typically ends with steam explosion, which is a convenient discharge method for a pressure reactor. Steam explosion efficiently reduces the particle size of the material.

1.4.2 Delignification methods

Delignification processes originate from the pulp industry, relying on the degradation and dissolution of lignin by alkaline or acidic chemicals, organic solvents or oxidative agents.[9] Alkaline delignification resembles the commercial process of soda pulping, which is the prevalent process for pulping non-wood feedstocks including agricultural residues and grasses.[81,82] These materials are easier to delignify compared to wood because of a lower lignin content and a high amount of ester linkages between lignin and carbohydrates.[54] Delignification leads to a very high enzymatic hydrolysability of the pretreated material and to glucose yields up to 80% with low enzyme loadings.[73,83]

Alkaline chemicals degrade lignin by break-down of aryl ether bonds and saponification of ester linkages. The degraded fragments are dissolved in the alkaline solution, which is facilitated by the deprotonation of the phenolic hydroxyl groups of lignin. The removal of lignin depends on the alkaline loading per dry matter, and a low loading can only partly be compensated by increasing reaction time or temperature.[83] In soda pulping, NaOH-loadings of 10–20% (per DM) are applied at temperatures between 160–180 °C for 1.0–1.5 h and the chemical is recycled from the black liquor after combustion of the dissolved lignin and other organic material.[81,84] From the perspective of saccharification, however, complete delignification is not required and the dissolution of hemicellulose must be minimized. Therefore, milder thermal severities (below 160 °C) and lower chemical loadings are applied. The frequently proposed alkaline chemicals include the hydroxides and carbonates of sodium, potassium and calcium, as well as ammonia.[83,85–88] Even the lowest effective chemical loadings (8% NaOH)[83] are still considered to require chemical recycling, which is costly. Locating the process near to an existing pulp mill has been suggested, but it is questionable whether the required extra capacity is available, since the chemical recovery boiler is often the bottleneck of a pulping process. An alternative is therefore further reduction of chemical consumption. Lignin is expected to provide a valuable side stream from biorefineries,[40] and instead of combustion, it can be recovered from the alkaline black liquors by acidification.[89–91]

Ammonia has particular potential, because it can be recycled by evaporation. It has been applied in low temperature soaking reactions,[92] ammonia recycle percolation (ARP)[93] and ammonia fibre explosion (AFEX)[87], which is a high temperature treatment with concentrated ammonia, followed by steam explosion and evaporation. In the AFEX-process lignin is not separated from the solids, and therefore it is not strictly a delignification method. However, it is an alkaline lignin-degrading process that facilitates cellulose hydrolysis by chemical modification of lignin, changes in the morphology of the material and decrystallization of cellulose. Ammonia consumption is estimated to be 3% of lignocellulose dry weight.[27]

Compared to acidic methods, no furans are formed in alkaline processes and the hydrolysates contain much less inhibitors compared to the hydrolysate from hydrothermal treatment. However, the dissolved lignin and the residual chemicals must be removed from the solids by washing and neutralization prior to enzymatic hydrolysis.

Organosolv-delignification was originally developed as a less polluting alternative for Kraft pulping, but with limited commercial success.[94] It has later re-emerged as a delignification method within the biorefinery context, with the particular potential of producing a higher quality lignin side-stream compared to the more degradative alkaline methods. Organosolv applies various organic

solvents, most commonly ethanol, at high temperatures for the dissolution of lignin. Acidic or alkaline catalysts are often added, and the solvent is recycled by distillation.[95–98] A recent study promoted a particularly high hydrolysability after delignification with acidified tetrahydrofuran, with over 95% glucose yield with low enzyme dosages.[99]

Oxidative degradation of lignin, in resemblance to pulp bleaching processes, is another delignification approach. Oxidative agents such as oxygen (wet oxidation), ozone or hydrogen peroxide are used for lignin removal alone or in combination with alkaline or organosolv treatments.[100–103] However, the oxidation approach suffers from the cost of the oxidative chemical consumption.

In order to improve the recovery of hemicellulosic sugars and to further improve the hydrolysability of cellulose, prehydrolysis with dilute acid or hydrothermal treatment has been proposed prior to different delignification methods.[104–107] However, only few reports have included overall sugar balances. An increase of hydrolysis sugar yield from 80% after dilute acid hydrolysis to 93% by subsequent organosolv-delingification has been reported.[104] In spite of potential yield improvements, such double-treatments have not advanced to demonstration scale.[27]

1.4.3 Flow-through processes

The majority of pretreatment studies are performed using batch reactors or co-current continuous reactors, which are essentially similar in terms of reaction kinetics. However, flow-through processes have been found to improve efficiency of different pretreatments. Particularly, flow-through has increased the dissolution of lignin in different delignification methods, including alkaline[106,108] and organosolv[96] treatments and ammonia recycle percolation,[109] as well as in hydrothermal pretreatment.[23,110,111] The reaction solution is pumped through a bed of lignocellulose material packed in a column, and the dissolved reaction products are washed away by the liquid flow during the reaction, thus reducing recondensation of lignin and degradation of sugars (Figure 5). The partially treated material is reacted with a solution with less dissolved products and in the case of alkaline delignification, with higher alkaline concentrations, which is beneficial for reaction kinetics and mass transfer. Theoretically, the pretreatment could even be followed by flow-through enzymatic hydrolysis in the same column.[108] The drawbacks of a percolation process are the non-continuous operation, the low throughput due to low packing density of lignocellulose and possible clogging of the flow due to the high compressibility of pretreated lignocellulose.[112,113] Percolation processes are also often associated with high water consumption.[8] On the other hand, in the case of straw, flow channeling is not expected to be a problem due to the high bridging tendency of the fibrous material.

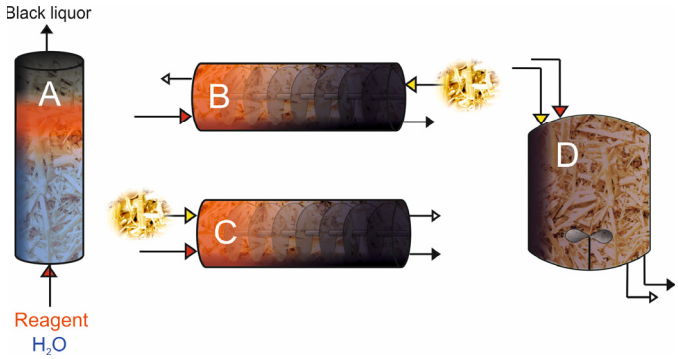

Figure 5. *Pretreatment reactors. A) Flow-through (percolation), B) counter-current flow-through, C) co-current reactor, D) stirred tank reactor (batch or continuous).*

Counter current operation has been expected to further improve the process.[96] Counter current flow enhances mass- or heat transfer between two phases, and is routinely applied in liquid-liquid extraction and heat transfer, as well as in washing Kraft pulp.[114] It is also found to enhance sugar yields in direct acidic hydrolysis of cellulose.[115] However, moving a solid substrate counter currently with liquid is difficult and only few reports of counter current pretreatments exist. These include a pilot scale autohydrolysis reactor[23] and a small extruder setup, which allows autohydrolysis and subsequent delignification in a single counter-current process[106]. Both reports lacked direct percolation as a reference, so the benefit of counter-current operation remains ambiguous. The counter current effect can also be achieved in a progressing cascade of columns operating as "progressing batch percolation".[115] However, no reports exist on its application for lignocellulose pretreatment.

1.4.4 Marginal pretreatment methods

Many exploratory approaches to pretreatment have been reported, including cavitation,[116] sonication,[117] microwaves[118] and electron beaming,[119] which typically show a small increase in hydrolysis degree with an unbearably high energy input or other costs. Better-established alternative pretreatment categories include mechanical and biological pretreatments and ionic liquids.

Practically all pretreatments include size reduction of the initial raw material by chopping or milling, but it is sometimes considered as a pretreatment itself. Size reduction and increasing the particle surface area are particularly important for the hydrolysis of wood materials.[120] For the thin particles of straw, however, size reduction is less effective, and the improvement of hydrolysability by mechanical treatments requires milling forces sufficient to decrease cellulose

crystallinity or to produce a very fine particle size. Practically this is only achieved at laboratory scale, typically by ball milling, and the required high energy input renders it inherently unfeasible.[121] The same applies to dry fractionation, which is a technology for separating the particles acquired by fine-milling.[121] Milling is required for logistical and technical purposes to increase packing consistency[113] and facilitating transportation by conveyors and feeding to reactors, but such coarse milling or chopping only has a small effect on hydrolysability.[122]

Biological delignification by fungi or bacteria has been studied as an environmentally friendly treatment. It could be performed locally by the lignocellulose providers, which enables long reaction times during storage, prior to transportation to the biorefinery.[9,123] Unfortunately, the lignin-degrading micro-organisms also consume part of the carbohydrates and improvements of hydrolysability are not comparable to thermochemical treatments.

Ionic liquids efficiently dissolve biomass and they can be used for fractionation of lignocellulose and decrystallization of cellulose, leading to efficient hydrolysis.[124] The use of ionic liquids, however, faces severe technical difficulties due to their price, toxicity and difficulties of recycling and removal. The residual ionic liquids in biomass lead to inactivation of cellulases, with only small improvement when thermo- or alkali-stable enzymes are used.[125,126] Additionally, many ionic liquids react with cellulose at the distillation temperatures, which hampers the already costly recycling.[127]

1.4.5 Direct dilute-acid hydrolysis

Direct acidic hydrolysis of cellulose has been known for a century and it is a competing approach to enzymatic hydrolysis.[5] It is a straightforward process and is not inhibitied by lignocellulose recalcitrance in the same way enzymes are, but it also harbors some drawbacks. Acidic hydrolysis is performed with sulfuric or hydrochloric acid at temperatures from 160 to 240 ^{o}C, and under such conditions, expensive corrosion resistant materials are required. Whereas practically all cellulose can be hydrolysed, the released glucose is also eventually degraded, which inherently restricts the sugar yield potential. From bagasse cellulose, after prehydrolysis of hemicellulose and alkaline delignification, 70 % glucose yields have been obtained with dilute sulphuric acid (~0.1% w/v).[128] Direct acid hydrolysis of straw has led to considerably lower glucose yields (47%), while xylose (79%) can be efficiently recovered by using a stepwise hydrolysis process.[129] Some improvement to the yields may be expected from flow-through and counter-current processes.[115] Nevertheless, the dilute acid hydrolysates are infused with sugar degradation products and phenolics, such as acid soluble lignin and related decomposition products, which are highly inhibitory to fermenting micro-organisms.[80,130] Finally, the acid catalyst must be

neutralized. Sulfuric acid is usually neutralized with calcium hydroxide, which at large scale leads to the accumulation of problematic amounts of gypsum that has to be disposed of. With hydrochloric acid, potential for acid recycling exists due to its volatility. However, enzymatic hydrolysis currently competes well with acid hydrolysis in terms of yield, quality and costs of sugars, as well as investments to the technology.[27]

1.5 Enzymatic hydrolysis of lignocellulose materials

1.5.1 Linear and non-linear hydrolysis responses

The hydrolysis of lignocellulosic carbohydrates is a complex multi-enzyme reaction that takes place on a heterogenous solid substrate. The hydrolysis is slow and the typical hydrolysis times range from one to six days. The hydrolysis rate is affected by decreasing hydrolysability of the substrate, as well as enzyme inhibition and denaturation, leading to asymptotic hydrolysis responses to time and enzyme dosage. The typical shape of hydrolysis time-curves are shown in Figure 6A. Even with very long reaction times, the maximum conversion that is being approached depends on the cellulase loading, suggesting that the enzymes are inactivated during the reaction.[131] This dependence is particularly visible with the lignin-containing materials from acidic pretreatments, compared to delignified materials, which better allow compensation of a lower enzyme dosage with increased reaction time.[132]The correlation of hydrolysis to enzyme dosage is also asymptotic, meaning that as the degree of hydrolysis increases, an increasing enzyme addition is needed for further improvement. In fact, the hydrolysis yield can be roughly linearized as a function of the logarithm of the enzyme dosage (Figure 6B).[133] This means that complete enzymatic hydrolysis can hardly be a feasible target.

Another important factor in hydrolysis is the consistency, *i.e*. the concentration of solids in the suspension. Given the complexity of the reaction, lignocellulose hydrolysis shows a surprisingly linear negative correlation to consistency (Figure 6C).[78] Although not fully explained, this has been suggested to result from mass transfer restrictions caused by increased viscosity, and increased product inhibition due to higher sugar concentrations.

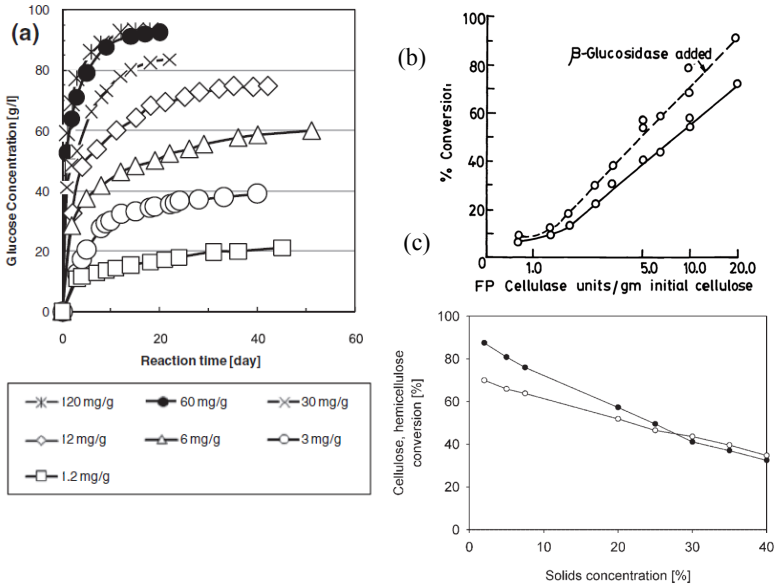

Figure 6. *A) Asymptotic time curves of lignocellulose hydrolysis with very long reaction time by Taneda et al.*[131§] *B) Logarithmic correlation of hydrolysis to cellulase dosage (Adapted from T. Ghose*[133]*) C) Linear correlation of hydrolysis of cellulose (solid circles) and hemicellulose (open circles) on solids concentration by Jørgensen et al.*[78**].

1.5.2 Enzyme adsorption, inhibition and denaturation

Cellulases are subject to different constraints during lignocellulose hydrolysis, and the most notorious nuisance is lignin. In addition to being a steric hindrance, lignin adsorbs cellulases, thus preventing them from accessing cellulose surfaces. The interaction of cellulases with lignin is strong and possibly irreversible,[14,134,135] but its effect on the activity of the enzymes is under debate. The lignin-bound cellulases are reported to retain most of their activity,[136] but lignin-mediated gradual inactivation of cellulases has also been suggested.[135]

Cellulases can also bind non-productively to cellulose. While the adsorption of cellulases on cellulose is generally reversible and fast,[61] unfavorable interactions also occur that cause binding of cellulases on the substrate, which restricts their action.[58] This is possibly related to accumulating irregularities on the cellulose surface during hydrolysis, as well as enzymes "jamming" each other at overcrowded adsorption sites.[58,137]

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Besides non-productive binding, product inhibition is a major obstacle. All cellulolytic enzymes are inhibited by glucose and xylose, and cellobiose and xylo-oligomers are particularly strong inhibitors to cellulases.[24,138] The inhibition affects the function of the catalytic domain as well as the cellulose binding module. Other enzyme inhibitors present in hydrolysis include lignin-derived soluble phenolics, formic acid and some ash components, depending on pretreatment.[130,139–141] The inhibitory effects vary between enzymes.[19]

Since the hydrolysis of cellulose is generally slow, the long reaction times may lead to denaturation of the enzymes. An optimal hydrolysis temperature is therefore a compromise between hydrolysis rate and thermal denaturation of the enzymes. Precipitation of cellulases in solutions has been reported already in the typical hydrolysis temperature range (45–50 °C),[142] but under actual reaction conditions, denaturation has been considered to have a smaller role, possibly because of stabilization of enzymes by binding to the substrate.[136,143] However, high mixing speeds have been reported to lead to negative effects due to shear-induced mechanical denaturation of the enzymes.[17,131] The typical pH-optimum for cellulases is between 4.5 and 5.0.

1.5.3 Hydrolysability of cellulose

The hydrolysability of the lignocellulosic substrate decreases in the course of hydrolysis[19]. According to the classic view, the more reactive cellulose with lower crystallinity, a lower degree of polymerization and better accessibility is hydrolysed first, leading to an increasing proportion of cellulose with a lower hydrolysability.[47,131,133] Unfavorable structures and irregularities such as fibril crossings and cellulose partly embedded in lignin may block the path of the advancing cellobiohydrolase.[58] However, detailed understanding of the reactivity change is still incomplete. Amorphous cellulose is hydrolysed faster than crystalline and therefore cellulose crystallinity was earlier considered to have a major role, but contrasting evidence on its importance on the final hydrolysis outcome has also emerged.[13,144,145] Another major factor is the cellulose surface area and its accessibility.[146–148] On the other hand, the non-productive binding on lignin depends on the accessible lignin surface area. More than 90% of the cellulose surface area is located within pores, and pores smaller than 5 nm are considered inaccessible for typical cellulases.[149,150] The pore network also constrains the diffusion of sugars, leading to increased product inhibition in small pores and large particles.[151] The hydrolysis of crystalline cellulose reveals fresh layers of cellulose molecules underneath, and thus the cellulose surface area may not follow a linear relationship with hydrolysis degree.[152] The pore structure is also changed by hydrolysis, further affecting cellulose accessibility.

1.5.4 Enzyme mixtures and hydrolysis additives

Lignocellulose hydrolysis is a result of synergistic action of cellobiohydrolases, exocellulases, endocellulases, cellobiases and xylanases and the optimal relative proportions of these enzymes depends on the material.[153] The hemicellulose composition particularly depends on material and pretreatment, requiring different hydrolytic activities in different cases.[154] Accessory enzymes such as feruloyl esterases,[64,65] acetyl xylan esterases[66] and pectinases[155] increase the overall hydrolysis yields. The use of lignin oxidizing enzymes, including laccases and peroxidases, has also been considered, but they have not gained wide application in lignocellulose saccharification.[65,156,157] Cellobiases are particularly needed in the beginning of hydrolysis, when the high hydrolysis rate leads to high cellobiose concentrations that cause product inhibition, while at later stages, a smaller dosage would be sufficient. Recently, attention has been paid to non-hydrolytic proteins called expansins, that loosen the cell walls of plants by reducing cellulose crystallinity (amorphogenesis), thus increasing cellulose availability to cellulases.[63] Another fresh research area concerns oxidoreductive enzymes known as lytic polysaccharide monooxygenases or LPMOs that depolymerize cellulose.[62] They work synergistically with conventional cellulases by oxidative cleavage of highly crystalline cellulose, producing end-oxidized cello-oligomers and free chain ends for cellobiohydrolases. Increasing attention is paid to LPMOs and they are gaining a role as another class of enzyme activities important for efficient cellulose hydrolysis.[158]

Many additives, particularly different surface active compounds and proteins, increase hydrolysis yields. Polyethylene glycol (PEG) has attracted most attention,[159] while other surfactants such as Triton X and Tween have also shown positive effects.[160] Similar effects have been obtained with bovine serum albumin (BSA).[161] The effect of these chemicals is attributed to the adsorption of these molecules onto lignin, reducing non-productive binding of cellulases by "blocking" lignin. Accordingly, the benefit is mainly observed with lignin-containing substrates, although reports of increased hydrolysis of microcrystalline cellulose (Avicel) by PEG addition also exist.[162] PEG has been shown to increase the proportion of soluble cellulases during hydrolysis, and to prevent precipitation of the enzymes.[142]

1.5.5 Product removal and enzyme recycling

Different approaches of product removal and enzyme recycling have been reported in order to reduce product inhibition and enzyme consumption.[21,24,163] In different "membrane reactor" configurations, ultrafiltration is applied to retain the soluble enzymes while removing the hydrolysate.[164] This is however considered to be a costly technology, considering the large hydrolysate volumes,

low enzyme concentrations and the small particles and impurities in hydrolysates which can block the membranes.[16] Sequential hydrolysis can be used as a simpler method for product removal, in which the hydrolysate is removed between hydrolysis steps and replaced with fresh liquid, while the majority of the enzymes are carried along with the solids.[165] This practically leads to dilution of the overall reaction conditions because of the addition of replacement water. Readsorption of the soluble enzymes from the hydrolysate to fresh substrate has been tested with some success, but this introduces sugars to the substrate, requiring extra washing.[143,166] A large portion of cellulases ends up in the solid hydrolysis residue, particularly with lignin-containing substrates. Recycling of these enzymes has been attempted by elution by alkaline or other chemicals, but this has led to extra chemical costs and poor enzyme recovery due to denaturation.[21,136] Another approach is coupling the enzymes to compounds that can be precipitated from the hydrolysate solution by changing the physicochemical conditions.[167]

It is known that mixing solid hydrolysis residue with fresh substrate leads to additional hydrolysis, suggesting that cellulases have been desorbed and relocated. This has led to the idea of enzyme recycling by recycling the solid residue.[166,168–170] Increased hydrolysis has been obtained in a steady state process of solids-recycling,[169] but it has not been conclusively shown whether this has been the result of actual enzyme accumulation or other effects. The increased reaction time of the recycled solids has been left undiscussed, and similarly to the reports of product removal by sequential hydrolysis, the overall water consumption or volumetric productivity is typically left uncontrolled when they have been compared to batch reactions. Reports on solids-recycling thus lack conclusions for the roles of enzyme recycling, product removal, reaction consistency and dilution.

1.5.6 Large scale lignocellulose hydrolysis.

The duration of the hydrolysis reaction is several days, and therefore vast reactor volumes are required for industrial scale hydrolysis. High solids concentrations, typically above 20% DM, are applied in order to increase volumetric productivity. Increasing the consistency of the reaction reduces the flowability of the slurry, which challenges mixing and material transfer.[171] Mixing is mandatory for efficient hydrolysis, but fortunately, a low mixing rate is sufficient, whereas high mixing speeds lead to excessive energy consumption and mechanical denaturation of cellulases.[17,78,172] The fibrous lignocellulose materials are efficiently liquefied during the early phases of hydrolysis in the first 3–6 hours. A prehydrolysis step has therefore been proposed in order to increase the solids concentration in the liquefied slurry. Suggested reactor types for prehydrolysis include a vertical gravitational plug-flow reactor[32] and a horizontal reactor with rotating scrapers facilitating free-fall mixing.[25,33,78] The

liquefaction reactor is then followed by a series of stirred tank hydrolysis reactors or SSF-reactors in cascade or in parallel.

On-site enzyme production is expected to reduce the cost of enzymes in an industrial scale hydrolysis plant. Production of enzymes next to the hydrolysis plant, potentially by a partnering enzyme producer, would allow direct use of enzymes with lower degree of purification, concentration and formulation, and the residual nutrients in the enzyme fermentation broth could be further exploited.[5,32,37] However, it has been estimated that the potential of the reduction of enzyme production costs is not alone sufficient for improving the process economy.[12] Decreasing enzyme consumption and valorization of the process side streams such as lignin are therefore key targets of research.[40]

1.5.7 Modelling of lignocellulose hydrolysis

Modelling is needed for process simulations as well as for scientific understanding of the process. A number of mechanistic models have been proposed for describing hydrolysis. An early approach to describe the hydrolysis rate dynamics was to differentiate between a discrete number of easy and recalcitrant parts of the substrate.[47] More recent models have typically incorporated adsorption kinetics and different rate-constraining effects as modifications to the Michaelis-Menten (MM) type kinetics.[18,173] The most comprehensive models typically have a high degree of complexity and number of parameters,[19,152] and simple empirical models are therefore considered better suited for process simulation purposes.[18] Mechanistic models are, however, an excellent tool for studying the theoretical background of lignocellulose hydrolysis. Although the classical method of studying initial hydrolysis rates has often been applied to lignocellulose hydrolysis, it fails to capture the hydrolysis-dependent effects such as changes in hydrolysability or in non-productive binding. Therefore it is necessary to apply complete hydrolysis time curves for model fitting.[19,152]

The NREL model by Kadam *et al.*[19] has received particular attention, since it thoroughly incorporates different known effects including product inhibition, reduction in substrate hydrolysability and adsorption of the enzymes on the solid substrate. The binding of the free enzymes $[E_F]$ on cellulose is assumed to follow the Langmuir isotherm (Eq. 1), where K_{ad} is the equilibrium constant of adsorption, $[S]$ is the amount of cellulose and e_m is the amount of binding sites on cellulose. The reaction rate r depends on the amount of bound enzymes $[ES]$ and the catalytic constant k_{cat}, and is restricted by product inhibition by different sugars I_i, including glucose, cellobiose and xylose, each of which have their own inhibition constant $K_{I,i}$. The hydrolysability of the substrate is assumed to decrease linearly as a function of hydrolysis degree, as described by the hydrolysability factor R_S (Eq. 3). Additionally, this set of equations is calibrated

$$[ES] = \frac{[S]e_m[E_F]K_{ad}}{1 + [E_F]K_{ad}} \qquad (1)$$

$$r = \frac{k_{cat}[ES][S]R_S}{1 + \sum \frac{I_i}{K_{I,i}}} \qquad (2)$$

$$R_S = \alpha \frac{[S]}{[S_0]} \qquad (3)$$

separately for at least exocellulases and endocellulases, and without adsorption and hydrolysability factors, to cellobiases. Although the NREL-model describes well the known effects in hydrolysis, it has 17 fitting parameters, which require a large amount of experimental data for fitting. Further studies of this model have shown that the parameters have poor identifiability due to redundancy, particularly because of the high number of parameters attributed to product inhibition.[20] On the other hand, the model neglects other possible mechanisms, such as permanent enzyme deactivation.

Another way of introducing a decreasing hydrolysis rate in the course of hydrolysis is fractal kinetics, which is suggested as an extension to the MM-kinetics and represents the effects of the heterogenous reaction, such as diffusion constraints.[137] The catalytic constant k_{cat} is replaced by a fractional rate constant kt^{-f}, which decreases over time according to a fractal exponent f. The phenomena behind the fractal rate constant have been loosely interpreted to represent the changing substrate hydrolysability as well as enzyme concentration and fractal kinetics is therefore regarded as an empirical model.[132]

More specialized models exist for the pore and surface accessibility,[151] cellulase adsorption,[174] shear induced deactivation[147] and enzyme jamming effects.[137] However, conclusion is yet to be reached on the phenomena that should be included, their representation and the balance between oversimplification and overparameterization.

1.6 Comparability of lignocellulose fractionation processes

The comparison of different fractionation processes is not straight-forward, because of the nonlinear responses of the hydrolysis yield to enzyme dosage and time, different energy, chemical and water consumptions and investment costs as well as different side streams and product quality. Previous technoeconomic analyses have often used fixed values for expected sugar yield and enzyme consumption of the process.[12,32,33,106] However, since enzyme consumption is a key cost, the feasible sugar yield target depends heavily on enzyme price.

Similarly, the investment cost for the hydrolysis reactor system depends on the residence time of the material. Optimizing the yield target with respect to enzyme dosage and hydrolysis time could provide a more accurate estimate of process feasibility and its sensitivity towards enzyme and equipment prices. More particularly, yield optimization can be expected to improve the process profitability estimates. In practice, this requires incorporating a yield response model into the feasibility calculations.

In the studies on hydrolysability of different lignocellulose materials, enzymes are often applied per pretreated dry matter. This, however, leads to incomparable total enzyme consumptions per untreated feedstock, due to the different dry matter yields of the different pretreatments. Applying the enzymes per cellulose better relates the enzyme consumption to the raw material feed, but this approach assumes that no cellulose losses occurred during pretreatment, which is not always accurate.[132] The mass balance has a similar effect on investment costs, since the reactor size required for a given hydrolysis time depends on the dry matter yield.

2 Aims of this study

This study focused on the pretreatment and enzymatic hydrolysis of wheat straw, which is a major agricultural residue in Northern Europe. The aim was to study different fractionation processes, describe the phenomena behind their effectiveness, find new process solutions and to advance the modeling of lignocellulose hydrolysis. A key driver was the need for reliable comparison of the potential of the major pretreatment categories, hydrothermal treatment and delignification. This provided simultaneous opportunities to improve the scientific understanding of the material properties affecting hydrolysability and to advance modeling of lignocellulose hydrolysis for process simulation as well as for mechanistic understanding of hydrolysis kinetics. The first target was to determine the relative importance of different factors affecting hydrolysability, including composition and surface properties of the material, pore accessibility and cellulose crystallinity. The second target was to study the changes in the material properties and hydrolysis rates as a function of hydrolysis degree. The third target was to combine this information with kinetic studies in order to determine the most important inhibition mechanisms that constrain the rate of hydrolysis.

In order to improve the efficiency and yield of lignocellulose fractionation, alternative process solutions were searched for. The applicability of a flow-through process for the pretreatment of straw was assessed and a comparison was made between counter-current flow-through, direct flow-through and batch reaction. In order to consider flow-through processes at large scale, the packing density, the required feed pressure and the risk of compaction of the material by the flow of liquid needed to be evaluated.

Enzyme recycling in hydrolysis was studied by recycling of the solid residue, and compared to product removal by sequential hydrolysis, with the emphasis on establishing conclusive comparability between the processes. Solids-recycling has been found to improve hydrolysis, but the evidence of enzyme recycling by this technique has been inconclusive. Clarification of the underlying mechanisms was therefore a subject of this study.

3 Materials and methods

This chapter briefly describes the most essential methods used in this study. For further details, the reader is referred to the original articles **I–V**.

3.1 Pretreatment and hydrolysis experiments

Wheat straw from Finland (35% glucan, 20.6% xylan, 58.6% total carbohydrates and 22.6% lignin and 4.5% ash) was chopped with a hammer mill to pass a 10 mm screen. Laboratory scale high pressure pretreatment reactions (**I** and **II**) were performed in a 200 mL Parr 4755 general purpose pressure vessel (Parr Instrument Company, USA) in a heated oil bath, and the reaction temperature was recorded using a Testo 175T3 data logger with a k-type thermocouple. The reactions were quenched by transferring the reactor into cool water. The material was filtered through a metal mesh and washed with excess water. For obtaining accurate mass balances, all materials remaining on the equipment were weighed and included in calculations.

The autohydrolysed straw used in **III** and **V** (54.1% glucan, 4.1% xylan, 0.3% arabinan, 27.4% lignin and 2.2 % ash) was produced in a stirred pressure reactor at 10% consistency by heating to 180–190 ^{o}C for 20 min, followed by steam explosion. Delignified straw (**III** and **V**; 78.7% glucan, 10.3% xylan, 0.7% arabinan, 3.8% lignin, 2.5% ash) was produced by a 5 h pre-extraction at 140 ^{o}C with water, followed by delignification with NaOH (38.6% of DM) under similar conditions.

Flow through delignification reactions (**IV**) were performed in 20 cm glass columns with a volume of 40 mL (Column C 16/20, GE Healthcare) in a convection oven at 90 ^{o}C. Straw was packed into the columns and liquid was fed through the straw bed using a Heidolph 5201 peristaltic pump (Heidolph, Germany). Dynamic pressure drop over the column was measured with a Testo 526 differential pressure meter.

The enzyme cocktail used in the experiments consisted by volume of 85% cellulase (Econase CE, AB enzymes), 10% cellobiase (Novozyme 188) and xylanase (GC140, Genencor). The total cellulase activity of the cocktail in filter paper units[175] was 51.0 FPU mL^{-1}, cellobiase activity in cellobiase units[174] was 65.4 CBU mL^{-1} and the protein concentration was 42 mg mL^{-1}. The conditions for hydrolysis, unless otherwise mentioned, were 50 ^{o}C, shaking at 200 rpm, pH

5 (50–100 mM sodium phosphate or sodium acetate buffer). To avoid microbial contamination, 4 mg mL^{-1} tetracycline and 3 mg mL^{-1} cycloheximide were applied. Reactions were performed in 5 ml Falcon tubes at 5 ml and Erlenmeyer flasks at volumes from 30 to 200 ml. Hydrolysis at solids concentrations above 15% was performed in a 5 L reactor with free-fall mixing by rotating scraping paddles (2 rpm), placed in an incubator (Figure 7).

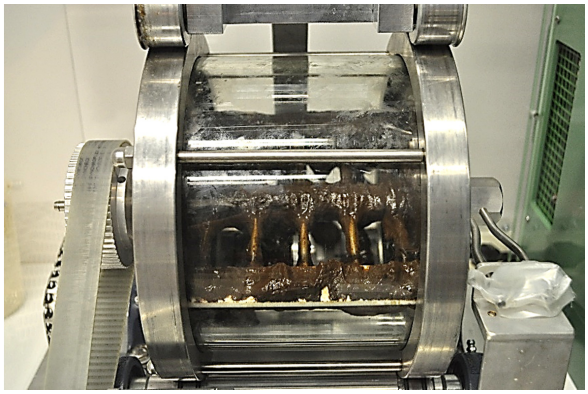

Figure 7. *Reactor for hydrolysis at high solids loadings, hydrolysing AH-straw.*

3.2 Analytical methods

Sugar concentrations were analysed with HPLC (Shimadzu, Japan), using a Shodex Sugar 08140 ion exchanger column (Shodex, Germany) in lead form and a Micro-Guard deashing precolumn (Bio-Rad 125-0118) at 60 °C with deionized water as the eluent at a flow rate of 0.7 ml min^{-1}. Monosaccharides and cellobiose were detected with RID 10A refractive index detector and quantified with Shimadzu Class-VP software and calibration with external standards.

Concentration of lignin in the black liquors from alkaline pretreatment reactions was analyzed using the 1260 Infinity high-performance size-exclusion chromatography (HPSEC) system (Agilent, Germnay) described previously,[107] with a series of three Ultrahydrogel columns (Waters, USA). The eluent consisted of aqueous 0.1 M $NaNO_3$ and 0.01 M NaOH, and the chromatography was performed at 30 °C at a flow rate of 0.5 mL min^{-1}. The VWD detector set (Agilent) at 280 nm was calibrated as a concentration detector for lignin using commercial wheat straw soda lignin (GreenValue lignin SA, Switzerland) dissolved in 0.2 M NaOH.

The surface area of cellulose was analyzed by determining the adsorption isotherm of the dye Congo Red[146,176] on the solid sample and determining the maximum monolayer adsorption capacity. The BET-isotherm was applied, as

described in **II**. The surface area of lignin, or more precisely, the amount of surface accessible phenolic hydroxyl groups, was determined with the adsorption method developed in our laboratory,[177] using the cationic dye Azure B and Langmuir isotherm. Non-specific binding to anionic carboxylic acid groups was ruled out by FTIR-analysis in **II**.

Pore size distributions of the solid materials were determined with DSC-thermoporometry (tpDSC). Thermoporometry relies on the melting point depression of small ice crystals confined within pores, which depends on the size of the crystals.[146,178] The volume of the pores of a given diameter thus corresponds to the volume of ice melting at a corresponding sub-zero temperature. The cumulative pore size distribution of the lignocellulose samples was measured with a DSC 6000 differential scanning calorimeter (Perkin Elmer), by determining the enthalpy change during stepwise heating of a frozen sample as described in **II**. Prior to analysis, the samples were thoroughly washed with deionized water to remove solubles.

The compressibility of straw was measured in triplicate by packing 368 g of straw in a scaled cylinder and recording the changes in the bed height by compression with a steel net piston, by stacking metal weights to up to 17.4 kg on top.

Compositional analysis of solid lignocellulose samples was performed according to the protocol by NREL[179], with a two-step hydrolysis with concentrated sulfuric acid. Oligomeric hemicellulosic sugars in solution were determined by hydrolysis with 4% sulfuric acid for 1 h at 121 ^{o}C. Dry matter of the samples was analysed either by oven drying at 105 ^{o}C or by lyophilization. Protein determination was performed spectrophotometrically with the BioRad protein assay (Bradford-method) against a BSA-standard. Dissolved phenolics (**III**) were quantified spectrophotometrically with the Folin-Ciocalteau method[180] against a gallic acid standard and expressed as gallic acid equivalent (GAE). Cellulose crystallinity was determined in the University of Oulu, by wide angle X-ray diffractometry, as described in **II**.

3.3 Modelling, statistical methods and calculations

All modelling was performed with Matlab R2010b (Mathworks). Nonlinear regression for model fitting was performed using *lsqcurvefit*.

The importance of different factors to hydrolysis in **II** was determined by principal component regression.[181,182] First, principal component analysis was performed to z-standardized variables **x** with *princomp.* Three components, accounting for 97% of the total variance, were included to the principal

component model as column vectors of **c,** and the model response $\hat{\mathbf{y}} = \mathbf{xc\beta_c}$ was linearly regressed with the hydrolysis results **y**, to give a linear regression coefficient for each principal component, $\boldsymbol{\beta}_\mathbf{c}$. The weight of each parameter was then calculated as the sum of their weights in the components, multiplied by their regression coefficients, $\boldsymbol{\beta}_\mathbf{x} = \mathbf{c\beta_c}$. Standard deviations of the $\boldsymbol{\beta}_\mathbf{x}$ were calculated from a bootstrap-distribution by fitting the principal component model separately into 1000 datasets of original size, obtained by resampling with replacement (discarding rank deficient sets, ~15%).

The kinetic hydrolysis models in **III** were fitted directly to hydrolysis time curves by nonlinear regression (*lsqcurvefit*) of the time integrals of the model, which were obtained by numerical integration using *ode15s*. To confirm global optimum of the fit, a three-level full factorial set of initial value combinations was produced and each combination was separately used by for fitting. To describe the explicitness of parameter estimation, the standard deviation was calculated for each parameter from the set of fitting results that reached 99% of the maximum coefficient of determination, R^2.

The straw packing density profile was simulated with Eq. 4, after fitting the parameters c_0, M and N to the measured correlation of density c (kg m^{-3}) as a function of compression pressure p_c (mbar). The dynamic pressure drop in an industrial scale column was simulated with the Kozeny-Carman model (Eq. 5 and 6), after fitting the specific surface area S^2 and the specific volume φ (m^3 kg^{-1}) to the measured pressure drop dp/dz (mbar m^{-1}) at different vertical flow rates v (m s^{-1}) and packing densities c. A value of 5.55 was used for the Kozeny-factor k, as previously suggested for fibrous materials.[183] A dynamic viscocity η of 0.315 mPas (water at 90 °C) was used. The void fraction is denoted by ε.

$$c = c_0 + Mp_c^N \qquad (4)$$

$$\frac{dp}{dz} = \frac{kS^2 v\eta(1-\varepsilon)^2}{\varepsilon^3} \qquad (5)$$

$$\varepsilon = 1 - c\varphi \qquad (6)$$

The severity factor for autohydrolysis, $\mathrm{Log}(R_0)$, was calculated as the 10-based logarithm of R_0, the time integral of the effect of temperature $T(t)$ (Eq. 7).[69]

$$R_0 = \int_0^t e^{\frac{T(t)-100}{14.75}} dt \qquad (7)$$

The "other costs" in the yield optimization simulation were calculated from the economic analysis by NREL[32] as follows: the feedstock and enzyme costs were subtracted from the reported minimum sugar selling price, which included a 10 year internal rate of return of 10%, corrected with 15% inflation (USA) since 2007. Dollars were converted to euros at an exchange rate of 0.922 euros per dollar.

4 Results and discussion

In order to compare the major pretreatment categories, hydrothermal treatment and delignification, an array of different fractionation processes were carried out and the mass balances were thoroughly analysed (4.1–0; **I**). The results were used to build an empirical model for the process sugar yields, which was used for the optimization and feasibility analysis of lignocellulose saccharification. This representative set of materials was further used to gain understanding of the factors behind enzymatic hydrolysability (4.3; **II**). Different physicochemical properties of the materials were determined and the magnitude of their effect on hydrolysability was determined. Next, the changes of the material properties during hydrolysis were studied, and different rate-constraining factors of hydrolysis were assessed by kinetic modeling (4.4–4.5; **III**). In order to improve the fractionation processes, different process solutions were tested. The effect of flow-through in delignification on hydrolysis was determined and the applicability of flow-through processes for straw was evaluated (4.6.1–4.6.2; **IV**). Finally, enzyme recycling and product removal during hydrolysis were studied by comparing recycling of the solid residue and sequential hydrolysis processes (4.6.3; **V**).

4.1 Autohydrolysis, delignification and their combination

The sugar yield potential of the two major pretreatment categories, autohydrolysis and delignification, was compared with wheat straw as the raw material (**I**). Autohydrolysis and NaOH-delignification were performed at three severities and the solid pretreated materials were hydrolysed with three enzyme dosages (4, 8 and 16 FPU g^{-1}) for up to 72 h. Autohydrolysis was performed at severities corresponding to the severity factor[69] $Log(R_0)$ of 3.6, 3.8 and 4.0, thus covering the previously reported optimum range.[23,68,107] For delignification, NaOH-dosages of 3, 6 and 12% of straw DM were used. Additionally, a double treatment was performed, consisting of autohydrolysis ($Log(R_0)$ = 3.8) and subsequent NaOH-delignification at three different NaOH dosages. The composition of the pretreated materials, their enzymatic hydrolysability and process sugar yields (16 FPU g^{-1}) are presented in Table 1.

Delignification led to a superior hydrolysability of the solids compared to autohydrolysis, with the highest glucan hydrolysability (85%) and sugar yield from straw carbohydrates (69%) obtained by direct delignification using a

Table 1. *NaOH-delignification, autohydrolysis and a double treatment with 3 different severities. Solids yield and composition, enzymatic hydrolysability as percentage of glucan and total carbohydrates in the pretreated material and process sugar yields as percentage of straw carbohydrates recovered in pretreatment, hydrolysis and altogether at an enzyme dosage of 16 FPU g^{-1}). Pooled standard deviation from the mean of duplicate compositional analysis or hydrolysis.*

	Solids yield	Composition, %			Enzymatic hydrolysability %		Process sugar yields %		
	%	Glucan	Xylan	Lignin	Glucan	Total	Pretreat.	Hydrol.	Total
NaOH 3%	78	41	23	20	47	50		44	44
NaOH 6%	68	49	24	14	72	76		66	66
NaOH 12%	55	57	26	8	85	87		69	69
AH 3.6 (Log(R_0))	80	44	20	23	41	44	11	39	50
AH 3.8	71	48	17	24	48	51	17	41	58
AH 4.0	70	51	12	25	52	54	21	41	61
AH 3.8 + NaOH	58	58	13	20	46	50	17	35	52
AH 3.8 + NaOH	48	65	12	15	53	56	17	35	52
AH 3.8 + NaOH	36	74	12	9	70	73	17	39	56
Untreated straw	100	35	21	23	14	13		13	13
Pooled standard deviation from mean	2.7	1.14	1.5	0.48	1.9	1.9	0.3	1.8	1.8

NaOH-loading of 12%. Only 61% total sugar yield was obtained by autohydrolysis, of which one third was comprised of the hemicellulosic sugars released in the pretreatment. Given the contribution of the hemicellulosic sugars to the overall yield and the better hydrolysability by delignification, it seems plausible that a double treatment could combine the benefits of both approaches, leading to maximal yields. As expected, the double-treated material was more efficiently hydrolysed compared to autohydrolysed straw. However, the double treatment led to the lowest sugar yield (56%), because of mass reduction and carbohydrate degradation. This serves as an example of the importance of the total mass balance over hydrolysability alone. A small overall improvement has previously been reported with the combination of dilute acid and organosolv treatments.[104] Accordingly, an increase in the final yield could still be expected with further optimization, but the improvement may not be large enough to warrant the extra costs of an additional reaction step. The same conclusion was previously made for a two-step autohydrolysis reaction.[25,79]

The hydrolysability of the NaOH-delignified wheat straw was in accordance with literature[184], as well as with the NaOH-delignified straw presented in **III** and **V** (see Appendix I). The yield of hemicellulosic sugars from autohydrolysis reached 21% of total carbohydrates, which is also at a similar level as previously reported.[23,68,105] The enzymatic hydrolysability of autohydrolysed straw was, however, lower than what has previously been reported[12,23], suggesting that even with the severity (Log(R_0)) of 4.0, the optimum for hydrolysability may not have been reached. Accordingly, 28–32% higher hydrolysabilities with similar enzyme loadings were observed for the autohydrolysed straw studied in **III** and **V,** corresponding to a hydrolysis yield of 52% with 16 FPU g^{-1}. For a fair

comparison between autohydrolysis and delignification, this higher hydrolysis potential is accounted for in the following considerations.

A linear increase in the degree of hydrolysis requires exponential enzyme addition[133] and therefore complete conversions cannot be expected at feasible enzyme loadings. The yield target must therefore be optimized with respect to enzyme consumption. A new way of illustrating this is presented in Figure 8, which allows yield optimization and accounts for the mass balances, establishing comparability of enzyme dosages or hydrolysis reaction times between different processes. The total enzyme productivity (mg sugars produced per FPU enzyme consumed in the complete process) is expressed as a function of the process sugar yield. This allows direct comparison between the processes (distance from the origin), and optimization of yield along a continuous curve for a given enzyme price and sugar value. Additionally, it roughly linearizes the correlation between yield and enzyme productivity, thus allowing projection towards theoretical maximum yields. Yield optimization curves can similarly be expressed for hydrolysis reactor volume, by plotting volumetric productivity of the reactor (kg total sugars m^{-3} h^{-1} hydrolysis volume) as a function of sugar yield, as demonstrated in **I**.

The original results (**I**) show higher enzyme productivity as well as yield potential for NaOH-delignification, compared to autohydrolysis. However, when the hydrolysability is assumed equal to the autohydrolysed straw in **III** and **V**, autohydrolysis show very similar results ("AH-potential") as NaOH-delignified straw, with slightly higher enzyme productivity below the yield of 62%. Extrapolation of the curves still promises a higher theoretical maximum yield for NaOH-delignified straw, but the difference is small.

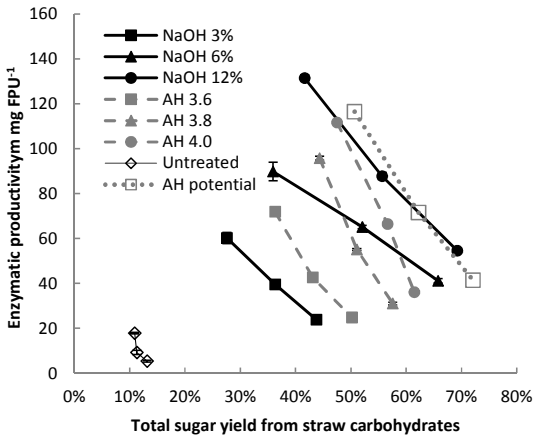

Figure 8. *Yield optimziation curves for enzyme consumption for NaOH-delignification, autohydrolysis and expected potential of autohydrolysis. The points represent results at 4, 8 and 16 FPU* g^{-1} *DM, respectively.*

While the above illustration allows intuitive comparison of the processes, accurate optimization and process simulation requires modelling of yield as a function of process parameters. A 2nd degree polynomial, frequently used in empirical modelling,[83,185] is not suitable for describing the asymptotic behavior[††] of hydrolysis and therefore a rational function model was built (Eq. 8) for the process sugar yield Y_{Tot} as a function of enzyme dosage E, time t and pretreatment severity S. The model describes the asymptotic behavior (Figure 9) with an excellent fit ($R^2 = 0.99$). Compared to a quadratic model, a smaller number of parameters is needed and more reliable extrapolation is possible. However, a quadratic model was applied for the dependence of the pretreatment sugar recovery Y_{AH} (Eq. 9) and the maximum hydrolysable carbohydrates $Y_{E,max}$ (Eq. 10) on pretreatment severity. The original parameter values are presented in the article **I**. However, the model parameters were re-calibrated for autohydrolysis to correspond to the hydrolysability of the autohydrolysed straw in **III** and **V** (see Appendix I). The model still shows a good fit ($R^2 = 0.98$), even though the results are gathered from several separate experiments (Figure 9B).

$$Y_{Tot} = Y_{E,max}\left(\frac{at}{at+1}\right)\left(\frac{bE}{bE+1}\right)\left(\frac{cEt+dE+et}{cEt+dE+et+1}\right) + Y_{AH} \quad (8)$$

$$Y_{E,max} = \alpha_1 S^2 + \alpha_2 S + \alpha_3 \quad (9)$$

$$Y_{AH} = \beta_1 S^2 + \beta_2 S + \beta_3 \quad (10)$$

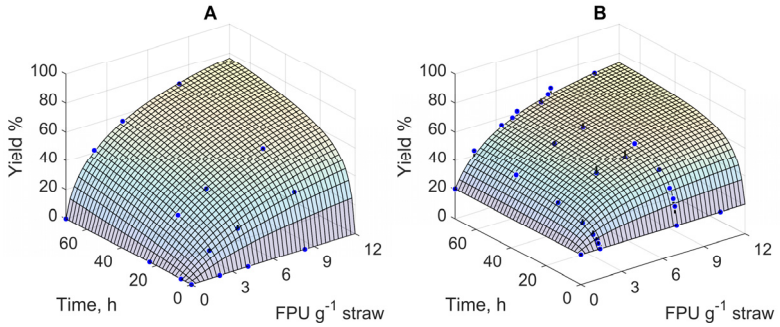

Figure 9. *Process sugar yield for A) NaOH-delignification (12% NaOH per DM) and B) autohydrolysis (Log(R0) 4.0) as a function of enzyme dosage and time. Response surface of the rational function model, averages of duplicate hydrolysis (dots) and the corresponding residuals (bars).*

[††] See supplementary material for **I**:
http://www.rsc.org/suppdata/gc/c4/c4gc02218a/c4gc02218a1.pdf

4.2 Economic Feasibility of the production of lignocellulosic sugars

The empirical model for process sugar yields presented above was applied when studying the dependence of the process feasibility on the price of cellulases and the market value of sugar. In addition to the comparison between autohydrolysis and delignification, two approaches for improving hydrolysis of AH-straw were evaluated, including the addition of the surfactant PEG and product removal during hydrolysis, according to the results in **V**, supported by some unpublished results (see Chapter 4.6.3 and Appendix I). The cost of straw was assumed to be 50 € ton^{-1},[7] according to the estimated cost of straw in Finland of 45–60 € ton^{-1} within a 10–100 km radius from the plant.[7] In the USA the price estimates of lignocellulose range between 37–74 € ton^{-1}, depending on demand.[29] A rough estimate of other process costs, excluding enzymes, was 97 € ton^{-1} straw. This figure was calculated from the NREL[32] technoeconomical analysis of ethanol and sugar production by dilute acid pretreatment and enzymatic hydrolysis of corn stover. It included both operational and investment costs as well as a 10-year internal rate of return of 10%. The costs of NaOH, PEG or additional process steps were excluded and the yield was simulated for a 72 h hydrolysis.

With the above mentioned estimates, the optimal yield target was calculated as a function of enzyme price (Figure 10A), assuming a value of the produced sugar equal to the 10-year average (2005–2015) of the sugar market price, 248 € ton^{-1}‡‡. At low enzyme prices, NaOH-treatment showed an increased yield optimum

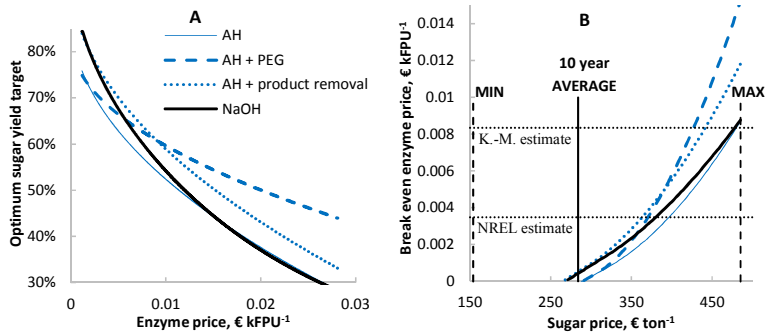

Figure 10. *A. The dependence of the optimal sugar yield target on the enzyme price, assuming a value of sugar equal to its ten-year average (2005–2015). B. The effect of sugar value on the enzyme price at which break-even return is reached in the production of sugar from lignocellulose. The vertical lines represent the ten year minimum, average and maximum values of sugar and the horizontal lines represent the enzyme price estimates from Klein-Marcuschamer et al.,*[12] *and NREL.*[32]

‡‡ CSCE contract No. 11. Source: Index Mundi, http://www.indexmundi.com/commodities/?commodity=sugar

compared to autohydrolysis, reflecting the higher maximum yield. Product removal shifted the optimum upward about 10 %-points compared to autohydrolysis, whereas PEG addition had a particularly high effect in the region of high enzyme prices, where the yield optimum was lower.

Next, the optimum yield target was determined as a function of both enzyme price and sugar value, and the break-even enzyme price was determined for each sugar value (Figure 10B). The cost of on-site enzyme production has been estimated to be 4.24 \$ kg^{-1} protein in the study by NREL[32] and 10.14 \$ kg^{-1} by Klein-Marcuschamer *et al.*,[12] which were converted to 0.0034 € $kFPU^{-1}$ and 0.0084 € $kFPU^{-1}$, respectively, according to the enzyme cocktail used in this work. According to both estimates, each of the processes would have been profitable for sugar production in 2011, when sugar price reached 480 € ton^{-1}. On the other hand, at the 10-year average sugar price, they would hardly be profitable even if enzymes were obtained for free. Looking at the relatively small differences between the process alternatives, it becomes clear that the sugar value makes a much larger difference than the improvements achieved by the addition of chemicals or process steps, even when the related additional costs are excluded. This highlights the volatility of the prospects of 2nd generation biofuels, and their dependence on factors external to process development. Nevertheless it can also be concluded that the processing of lignocellulosics has true potential for profitability even for sugar production alone, although the market price for food grade solid sugar is an overestimate for the obtained dilute aqueous mixtures of glucose, xylose and impurities.

While the process economy may be boosted by subsidies and mandates, it is essential to find extra value for the process side streams, particularly lignin. The majority of lignin in autohydrolysed straw is recovered in the solid hydrolysis residue, but it is associated with some residual carbohydrates, although a part of the lignin could be recovered from the autohydrolysis liquor.[89,90] Delignification methods provide a better opportunity for the recovery of high-purity lignin from the black liquor by acidification. For example, lignin from Kraft-pulping has been recovered by using the CO_2 of the flue gases of the process.[91,186,187] Potential lignin-based products include a range of aromatic platform chemicals, phenolic resins, biomaterials and fuels.[40,188,189] Although the search for applications and processes for the valorization of lignin is still underway, it could provide the missing piece for the feasibility of lignocellulosic renewables.

4.3 Factors behind the hydrolysability of lignocellulose

Composition, accessibility and cellulose crystallinity are the major lignocellulose properties contributing to hydrolysability and recalcitrance, and

they have been frequently characterized for different samples separately or in parallel. However, the relative importance of the factors remains under debate.[13,190] Pulling the open ends together, this work presents the first statistical analysis of the relative importance of the major factors (**II**). First, different properties of the delignified, autohydrolysed and double-treated materials presented in **I** were analysed, including cellulose surface area, accessible phenolic hydroxyls on the lignin surface, pore size distribution and cellulose crystallinity. Then these properties were correlated with enzymatic hydrolysis with 20 FPU g^{-1} glucan for 72 h (Figure 11). There appeared to be linear dependence between some of the variables and therefore linear regression could not be used to determine the weights of the different factors. Instead, principal component regression was used, which allows reduction of the collinearity.[181,182] Each factor was thus allocated a β-value representing its weight in the hydrolysis response. The six studied factors are presented in the order of importance. For the determination of the β-values, the lignin and xylan content were defined as proportional to cellulose content. For clarity, the β-values are converted into weight-% of the total response (% of the sum of the absolute values of β:s).

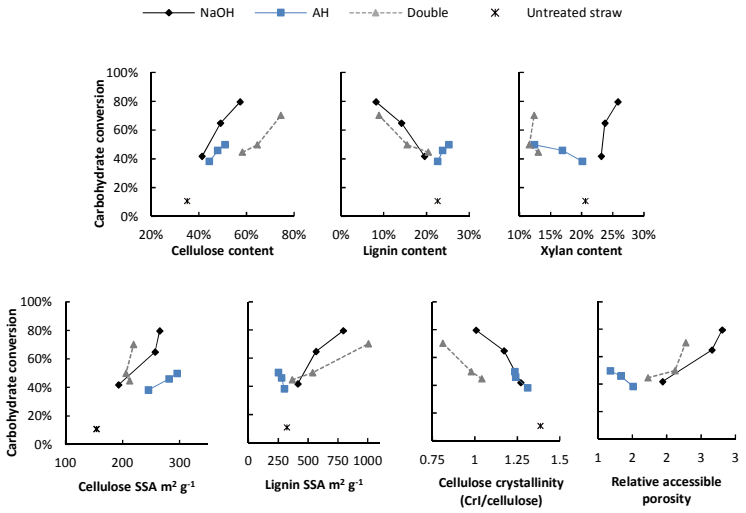

Figure 11. *Correlation of hydrolysis (20 FPU g^{-1} cellulose) degree with different material properties. The relative accessible porosity of native straw was excluded as an outlier, resulting from effects outside the scope of this study.*

1. Specific cellulose surface area, β = 29 %

Specific cellulose surface area was the most important factor facilitating hydrolysability, highlighting the importance of the accessibility of cellulose to cellulases.[146] The cellulose surface areas were determined by dye adsorption (Figure 12). Autohydrolysis most efficiently uncovered the surface of cellulose, possibly reflecting the removal of hemicellulose directly associated with cellulose[55]. Direct delignification was also effective, but when autohydrolysed straw was delignified in the double treatment, the specific cellulose area was decreased. This may be the result of lignin condensation products blocking cellulose surfaces[82], or aggregation of cellulose fibres in the absence of hemicellulose and lignin[45,191]. The latter is in agreement with the observed widening of the small pores of the material, as presented below.

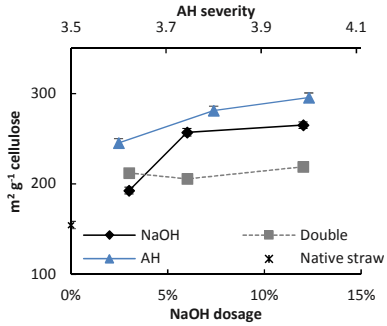

Figure 12. *Effect of pretreatments on cellulose SSA*

2. Relative pore accessibility, β = 23%:

While the cellulose surface area represents the local accessibility of cellulose, the accessibility also depends strongly on the pore structure of the material, since 90% of the lignocellulose surface area is located within pores[149]. The pore size distribution in each material was determined by DSC-thermoporometry in the pore diameter range from 1 to 396 nm (Figure 13). The relative pore accessibility was defined as the ratio of the pore volumes over and under the cut-off of 10 nm,

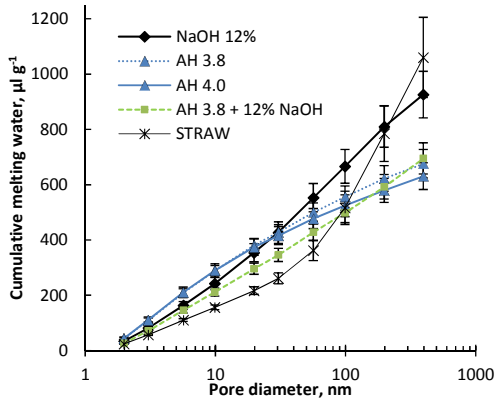

Figure 13. *Pore size distribution of wheat straw before and after pretreatments.*

and was found to better correlate with hydrolysability than the total accessible volume. Pores under 5–10 nm in diameter have been considered inaccessible for cellulases.[150,192] All pretreatments increased the volume of the pores below 100 nm in diameter, and the porosity was particularly increased by lignin dissolution in direct delignification, whereas hemicellulose dissolution in autohydrolysis led to a decrease in the overall porosity. After hemicellulose was dissolved in autohydrolysis ($\mathrm{Log}(R_0)$ 3.8), only minor changes in porosity were observed by delignification in the double treatment. It thus appears that hemicellulose actually contributes positively to hydrolysis by maintaining the porosity of the cellulose fibre network.

3. Lignin content, β = 17%

Not surprisingly, the mere presence of lignin turned out to be the largest negative factor and the removal of lignin was the most obvious positive result of the NaOH-treatments. Autohydrolysis does not significantly remove lignin, which poses an inherent constraint for the sugar yield potential by this process.

4. Phenolic hydroxyls on lignin surface, β = 16%

The specific lignin surface area in the materials was determined by cationic dye adsorption (Azure B), which binds to the phenolic hydroxyls of lignin. However, considering the variable surface chemistries resulting from pretreatments, the amount of phenolic hydroxyls may better describe the hydrophilicity of the lignin surface than the absolute area. The amount of phenolic hydroxyls per lignin was increased by the NaOH-treatments (Figure 14), which is known to result from the alkaline cleavage of the aryl ether bonds of lignin.[82] A similar effect was observed with ammonia in our previous study.[67,148] The increase in phenolic hydroxyls had a positive effect on hydrolysis. Therefore, in addition to lignin removal, the NaOH-treatments appear to make lignin less harmful to hydrolysis. Autohydrolysis slightly decreased the amount of accessible phenolic hydroxyls, which could result from the formation of pseudo-lignin condensation products[71,193] and reduction in surface area by relocation and coalescence of lignin,[70] which is known to occur in hydrothermal pretreatments. Hydrophobic interactions play a significant role in the non-productive binding of enzymes, but a suitable pattern of phenolic hydroxyls is also considered important.[14,194] Hydrolysis therefore benefits from both an increased hydrophilicity of lignin[195], particularly by charged groups[196,197], as well as the alkylation of the phenolic

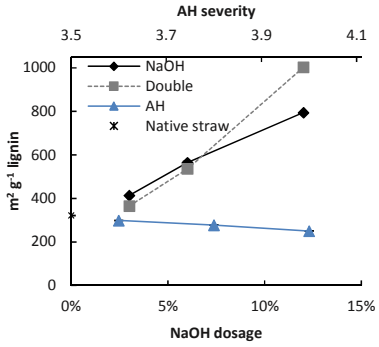

Figure 14. Effect of pretreatments on accessible phenolic hydroxyls on lignin (specific surface area of lignin).

hydroxyls[194,198], which in turn increases hydrophobicity. This reflects the potential of lignin surface modification for promoting hydrolysis, either covalently or with surfactants.

5. Cellulose crystallinity, β = 11%

Cellulose crystallinity was the second negative factor for hydrolysis and it was decreased by all pretreatments, particularly the NaOH treatments, according to determination of the crystallinity index (CrI)[199] by X-ray diffractometry (Figure 15). However, the effect of crystallinity was not drastic for the final conversion yields, and its effect was more pronounced in the beginning of hydrolysis, reflecting the fast initial removal of amorphous regions of cellulose.[144] Debate exists on the role of crystallinity and its analysis. The amorphous cellulose has been suggested to be rapidly hydrolysed, but on the other hand, only minor changes in the proportion of crystalline cellulase have been observed during hydrolysis.[13,144] Pretreatments are generally considered to disrupt the hydrogen bond network of cellulose crystals,[93,190,191] although harsh pretreatment conditions have sometimes led to an apparent increase in cellulose crystallinity[200] due to degradation of the amorphous regions. Due to known inaccuracies in the applied CrI determination method,[145,191] the presented CrI/cellulose ratio represents a relative index, rather than actual percentage of cellulose crystallinity. However, the apparent conclusion is that pretreatments decrease crystallinity, which positively affects hydrolysis. Although it is clear that amorphous cellulose is more efficiently hydrolysed by enzymes, the majority of cellulose remained crystalline in these conventional pretreatments and therefore, the effect of decreasing crystallinity was relatively small for the final conversion.

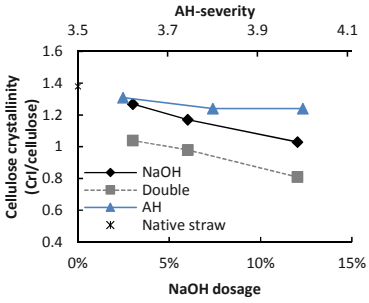

Figure 15. *Effect of pretreatments on cellulose crystallinity.*

6. Xylan content, β = 4%

The effect of hemicellulose on hydrolysability was small. Although the initial glucan hydrolysis was constrained by xylan (see article **II**), the final hydrolysis yield was not negatively affected. On the contrary, xylan had a small positive effect, which may be related to its contribution to the accessibility of the pore structure, as discussed above. A mild pretreatment is enough to allow hemicellulose to be efficiently co-hydrolysed with cellulose and it thus comprises a significant part of the sugar yield. In accordance with previous literature[71,201,202], xylan hydrolysability coincided with deacetylation, both of

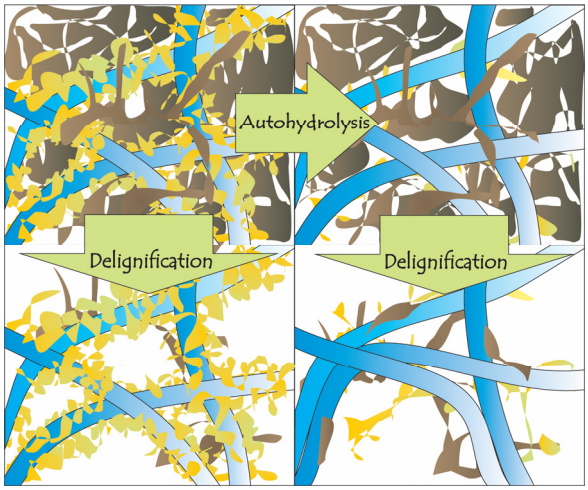

Figure 16. *Effects of pretreatments to native straw (top left). Cellulose fibrils (blue), hemicellulose (yellow) and lignin (brown).*[§§]

which were efficiently achieved with all pretreatments except with the lowest (3%) NaOH-dosage in direct delignification (**I**).

A summary of the above findings is illustrated in Figure 16. Dissolution of hemicellulose by autohydrolysis efficiently uncovers the surface of cellulose, but this is counteracted by a high lignin content and restricted accessibility, thus constraining the sugar yield potential. Direct delignification removes the burden of lignin and leaves a porous cellulose-hemicellulose network with excellent hydrolysability. However, if hemicellulose is removed prior to delignification, the network structure collapses and a part of the accessible cellulose surface is lost due to aggregation of the fibrils,[45,191] leading to decreased hydrolysability. Accompanied by increased sugar degradation in the double treatment, the sugar yields of the overall process were not competitive in comparison to direct delignification.

4.4 Changes in hydrolysis rate and material properties during hydrolysis

The change of hydrolysis rate in the course of the reaction was studied and compared to the changes of different material properties, with the aim to signify rate constraining factors. A simple experiment displayed the typical kinetic

[§§] Reproduced from **II** with permission from the Royal Society of Chemistry.

behaviour of lignocellulose hydrolysis (Figure 17A). In the hydrolysis of autohydrolysed straw, nearly half of the hydrolysis achieved with 10 FPU g^{-1} was reached with an enzyme dosage of 2 FPU g^{-1}, showing nonlinear dependence of hydrolysis on enzyme dosage. Also, the two reactions approach different asymptotes, as if the amount of the catalyst would affect the chemical equilibrium, which is not expected from catalysis.[131,133] A closer look at the hydrolysis rates as a function of hydrolysis degree (Figure 17B)

reveals that after increasing the 2 FPU g^{-1} dosage to 10, an equal hydrolysis rate is immediately observed and an equal hydrolysis degree is reached as by directly applying 10 FPU g^{-1}. This allows some direct conclusions. First, the hydrolysis rate depends exclusively on the degree of hydrolysis and no effective time-dependent changes occur in the substrate, other than hydrolysis itself. It was first hypothesized that physical changes in the morphology of amorphous lignin may cover fresh cellulose surfaces, which could lead to higher inhibition of the slower reaction, but this possibility is overruled by the results. It is also clear that there is absolutely no benefit in stepwise enzyme addition in a simple batch hydrolysis. Second, the hydrolysability of the substrate is substantially reduced. The hydrolysis rate at 10 FPU g^{-1} after 20% hydrolysis was several times lower than the initial rate and only half of the initial rate observed with 2 FPU g^{-1}. The rate reduction is too drastic to be explained by product inhibition alone.[24] Accordingly, the inhibitory properties of the hydrolysates were tested at end concentrations and found to lead to no more than 40% inhibition. Third, in accordance with previous hydrolysis reports,[136,143] the results do not indicate time-dependent enzyme denaturation, since similar hydrolysis rates are observed after different exposures of enzymes to hydrolysis conditions in the stepwise and direct 10 FPU g^{-1} reactions. Additionally, the residual soluble cellulase activities were determined and found to decrease in linear correlation with hydrolysis degree, rather than time.

To gain further understanding of the kinetics of hydrolysis and factors constraining it, different physicochemical properties were determined as a function of hydrolysis degree, the foremost being the surface areas of cellulose

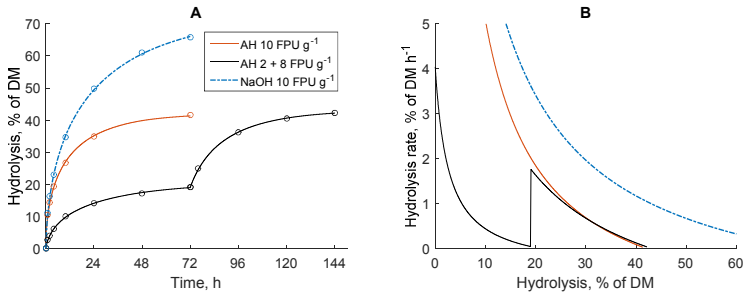

Figure 17. *A) Hydrolysis degree as a function of time. B) Rate of hydrolysis as a function of hydrolysis degree (**III**).*

and lignin. Reaction rate depends on the substrate concentration [S] and the cellulose surface area has been stated to better represent the substrate concentration available for cellulases than the total cellulose content.[19,152] However, the cellulose surface area was found to decrease linearly with the hydrolysis degree, signifying that the total cellulose amount is a decent relative approximation of [S] after all, and thus applicable for kinetic studies (Figure 18). On the other hand, the surface area of lignin, responsible for the non-productive binding of cellulases,[135,203] was initially decreased. This was explained by the dissolution of phenolics, which was facilitated by hydrolysis. The contribution of enzyme adsorption to the decrease in the detected lignin surface is expected to be small and the magnitude of the decrease did not correlate with enzyme dosage (**III**). After the initial decrease, only minor increase was observed as the hydrolysis advanced, indicating that most of the lignin surface is already exposed initially, and hardly any fresh lignin surfaces are revealed during hydrolysis. Hydrolysis could nevertheless increase the accessibility of the lignin surfaces, thus facilitating a form of "product inhibition" by increased adsorption on lignin. The accessibility of the materials during hydrolysis was studied by measuring the pore size distribution using tpDSC. However, no conclusive trends could be distinguished for autohydrolysed straw within the error limits, while a small decrease in porosity was observed with NaOH-delignified straw. Previously, somewhat decreasing cellulose accessibilities have been reported during hydrolysis for alkali and acid pretreated lignocellulose. Further clarification is thus required to reach a conclusion on the changes on cellulase-binding potential of lignin during hydrolysis.[204]

The only effect that was found to be partly time-dependent was the dissolution of phenolics, but this did not have an effect on substrate hydrolysability. Also,

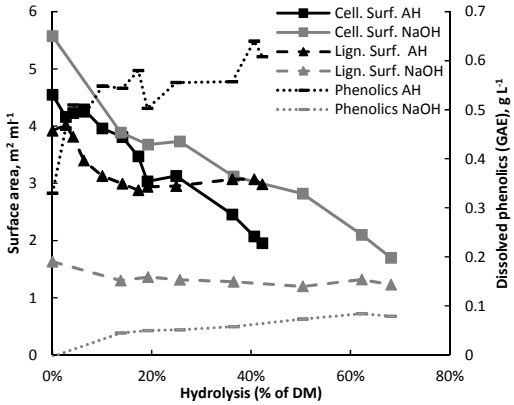

***Figure 18**. Cellulose and lignin surface areas and phenol dissolution as a function of hydrolysis degree of autohydrolysed and NaOH-delignified straw.*

the concentration of the phenolics was low and did not cause observable inhibition of cellulases. More particularly, the hydrolysate liquors separated after enzymatic hydrolysis of autohydrolysed and NaOH-delignified straw showed little inhibitory effects beyond those by a pure mixture of glucose and xylose at equal concentrations, signifying that enzyme inhibitors were not released by hydrolysis in effective amounts. The release of inhibitory compounds is thus an issue in the pretreatment,[80,130,139] but not in enzymatic hydrolysis.

4.5 Assessment of inhibitory effects by kinetic modelling of hydrolysis

Different inhibition scenarios associated with lignocellulose hydrolysis were studied by kinetic modelling. The model was simplified as a single enzyme reaction by assuming processive hydrolysis of cellulose as the rate limiting reaction, following the reaction equation Eq. 11. The basis of the model presented in **III** was similar to the NREL-model by Kadam *et al.*[19], assuming Langmuirian adsorption of the enzymes, and a first order reaction rate for the adsorbed enzymes. As an improvement, the adsorption equation was solved for the concentration of enzyme-substrate complexes [ES] and could therefore be fitted without separately determining adsorption kinetics of the enzyme. Different inhibitory factors were incorporated to this model separately and in combinations.

The difference of this model from the classic Michaelis-Menten equation is that in the MM-equation, it is assumed that $[S] \approx [S_{tot}]$, thus excluding the degree of saturation of substrate binding sites. In Langmuirian adsorption, the free or bound sites are accounted for and thus $[S] = [S_{\mathrm{tot}}] - [ES]$. However, it was observed that the equilibrium constant of adsorption K_{ad} and the amount of binding sites in cellulose e_m were poorly identifiable[***], suggesting that the saturation of the substrate binding sites was not substantial enough to significantly affect hydrolysis. In this work, the modelling was partly repeated without including the Langmuirian adsorption, thus leading to an equation analogous to the MM-equation (Eq. 12). This generally improved the accuracy of parameter determination, without compromising fit (Figure 19, Table 2). In this form, K_{ad} and e_m are linearly dependent and thus lumped into a single parameter K (Eq. 13), reducing the number of parameters by one. Due to the processive action of cellobiohydrolases, enzymes are not released from the substrate simultaneously with the product and therefore k_{cat} does not appear in K, which thus differs from the classic MM-constant.

[***] See Additional file for **III,** available at:
http://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-016-0431-3

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_{cat}} P \qquad (11)$$

$$r = \frac{k_{cat}[E_0][S]}{K + [S]} \qquad (12)$$

$$K = \frac{k_{-1}}{k_1 e_m} \qquad (13)$$

If inhibitory factors are assumed to arise as a linear function of hydrolysis, their amount can be described by Eq. 14. This applies to reversible (competitive) product inhibition by sugars (I_{rev}), as well as possible irreversible inhibitors released by hydrolysis (I_{irrev}). The lignin surface can also be seen as an inhibitor, but it is not clear whether binding on lignin is strictly reversible or irreversible and how lignin accessibility depends on hydrolysis. The hydrolysability of the substrate is known to decrease during hydrolysis, which can be described as a reduction of k_{cat} by the hydrolysability factor I_h. However, the linear relation to hydrolysis degree, proposed in the NREL-model[19] is only the simplest arbitrary assumption.

$$I_i = \frac{\alpha_i([S_0] - [S])}{[S_0]} \qquad (14)$$

Irreversible product inhibition leads to a decrease in enzyme amount $[E_0]$ from the initial $[E_{0,init}]$ by the amount bound to the inhibitor, $[EI_{irrev}]$. Linearly increasing lignin accessibility could be described this way. If irreversible inhibition is assumed to be "instant" compared to the long hydrolysis times, then $[EI_{irrev}] = I_{irrev}$. However, if the irreversible inhibition is considered to occur gradually, then a rate equation for the formation of $[EI_{irrev}]$ applies (Eq. 15). This, for example, covers the case of gradual denaturation of enzymes on lignin surface, which has been suggested previously,[135] combined with increasing lignin accessibility by hydrolysis. In this case, relating I_{irrev} to hydrolysis degree corresponds to the assumption that accessible lignin surface is linearly increased as a function of hydrolysis degree. On the other hand, if the accessible lignin amount remains constant, the rate equation becomes analogous to that of (thermal) denaturation. This would be in accordance with the negligible increase of lignin surface observed during hydrolysis. Since each of these options gave a good fit in combination with reversible product inhibition and decreasing hydrolysability, more data is required for further elucidation between them.

$$\frac{d[EI_{irrev}]}{dt} = \lambda([E_0] - [ES])(I_{irrev} - [EI_{irrev}]) \qquad (15)$$

It was concluded in **III** that reversible product inhibition and reduction of hydrolysability are both known to occur, but neither could explain the dependence of the hydrolysis maximum on the enzyme dosage (Figure 19A). Therefore, an irreversible form of inactivation is required in the model, either by denaturation or irreversible product inhibition. The permanent effects alone lead to a reduction in enzyme amount that is too drastic, leading to a complete depletion of the smaller enzyme dosage (Figure 19B). However, in combination with the other effects, an excellent fit was obtained (Figure 19C). It thus seems justified to include product inhibition, degreasing and a permanent effect in the kinetic model, before adding more details to either of them, as proposed in Eq. 16. This model can be considered an improvement to the NREL-model[19], with the factors better balanced. The MM-type models also have a better theoretical background than the recent fractal model for hydrolysis, which assumes a time-dependent change in the rate constant.[132] In the future, further characterization

$$r = \frac{(k_{cat} - I_h)([E_{0,init}] - [EI_{irrev}])[S]}{K(1 + I_{rev}) + [S]} \qquad (16)$$

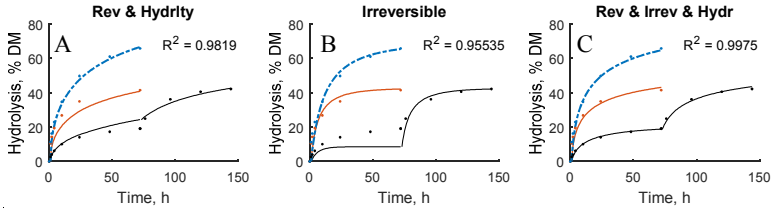

Figure 19. *Kinetic hydrolysis models compared to actual datapoints. A) Reversible product inhibition and reduction of hydrolysability. B) Irreversible product inhibition. C) Combined effect of irreversible and reversible product inhibition and reduction of hydrolysability.*

Table 2. *The re-fitted kinetic parameters for different combinations of reversible and irreversible inhibition and reduction of hydrolysability as rate-constraining factors. Parameter standard deviations from mean in parenthesis.*

Rate-constraining factors	R^2	K mL/FPU	$k_{cat,AH}$ mg $FPU^{-1}h^{-1}$	$k_{cat,NaOH}$ mg $FPU^{-1}h^{-1}$	α_{Rev}	α_{Irrev} FPU ml^{-1}	α_{Hydr} mg $FPU^{-1}h^{-1}$
No inhibition	0.844	4315 (45%)	281.3 (45%)	254.5 (45%)			
Irreversible	0.955	5673 (68%)	1642 (66%)	866.6 (66%)		0.680 (0.2%)	
Reversible & hydrolysability	0.982	1.592 (141%)	144.08 (78%)	148.2 (77%)	3896 (140%)		73.97 (73%)
Reversible & irreversible & hydrolysability	0.997	0.0482 (129%)	15.46 (109%)	13.41 (102%)	4240 (198%)	0.283 (1%)	13.69 (75%)

of the inhibitory factors should determine whether or not they correlate linearly with hydrolysis degree, as well as whether the interactions with lignin are fast enough to be considered instantaneous.

4.6 ALTERNATIVE PROCESS CONFIGURATIONS

4.6.1 Delignification as a percolation process

Flow-through delignification of a straw bed packed in a column was studied as direct percolation and as a counter current process (progressing batch percolation[115], as described in **IV**). The reactions were performed with low NaOH-loadings (3–6% of straw DM) at 90 °C at atmospheric pressure. The volumes of the NaOH-feed corresponded to reaction consistencies of 10, 20 and 40% DM (10% for batch reactions), and the delignified straw was washed with water (displacement washing for flow-through and suspension-washing for batch reactions).

Compared to batch reactions, the flow through processes improved the dissolution of lignin as well as enzymatic hydrolysability, in accordance with literature.[106,108,109] In fact, the sugar yields from the flow through process were almost equal to those achieved with the pressurized batch reaction described in Chapter 4.1 (**I**), where much more severe thermal conditions were applied (140 °C for 1 h) at equal NaOH-loadings (Figure 20). Hemicellulose dissolution and NaOH-consumption were not affected by flow-through operation.

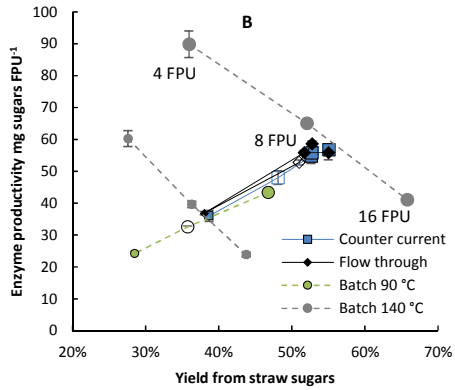

Figure 20. *Comparison of the hydrolysis yield and enzyme productivity of the flow-through and batch processes at 90 °C (**IV**) to the yield optimization curves of the batch reactions at 140 °C presented in Chapter 4.1 (**I**). Small, open and large markers represent NaOH-loadings of 3, 4.5 and 6%, respectively.*

Contrary to expectations, counter-current operation did not further improve the process compared to direct percolation.[96,115] The effectiveness of flow-through is attributed to the washing effect, removing dissolved lignin from the reaction and thus reducing recondensation and further degradation. However, it appears that the washing effects are already achieved with direct percolation, *i.e.* when the liquid and solid phases are moving relative to each other, regardless of whether the solid phase is moving or stationary. However, counter-current reaction provides a way to perform a flow-through reaction as a continuous process, if suitable equipment are available.[23,106,114]

4.6.2 Operability of a flow-through pretreatment in industrial scale

In order to assess the industrial scale applicability of flow-through pretreatments, the compressibility and flow properties of untreated and delignified straw were determined. The packing density and the required feed pressure for an industrial scale column were simulated up to 20 m bed height (Figure 21). In a 10 m column, the packing density of fresh straw increases from 103 kg m^{-3} at the top to 157 kg m^{-3} at the bottom, unless mechanical compression is applied for packing. These densities were higher compared to the previously reported densities up to 73 kg m^{-3} for uncompressed wheat straw, possibly due to a smaller particle size.[112,113] It was found that the dynamic pressure drop of the flow must be maintained below 1 bar m^{-1} in order to avoid clogging by compaction of the delignified straw bed. This restricts the maximum applicable feed pressure, which determines the corresponding minimum operation time needed for feeding the reaction liquor and washing water into the column. The maximum applicable feed pressure for a 10 m column was 5.5 bar. Correspondingly, a reaction at 20% consistency and washing with one column volume of water requires a minimum operation time of 50 min, thus setting the maximum straw throughput to 163 kg $m^{-3}h^{-1}$ (excluding downtime for discharge and packing). If the column height is increased to 15 m, the operation time is

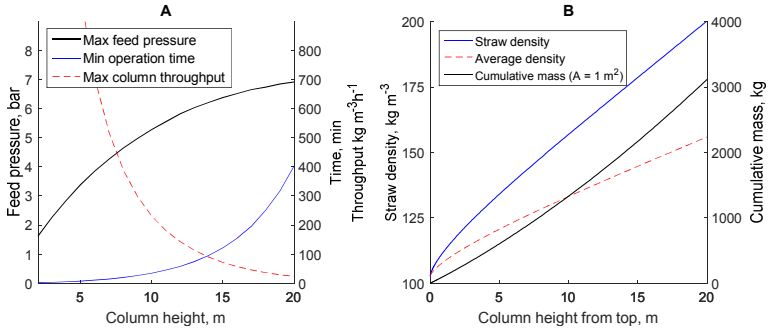

Figure 21. *A. Maximum applicable liquid feed pressure through a straw bed as a function of bed height, and the corresponding minimum operation time and straw throughput. B. Straw bed packing density profile, average bed density and straw load as a function of bed height.*

almost tripled, thus decreasing the straw throughput, even though the packing density is increased. Nevertheless, it is concluded that flow-through operation is applicable for straw in industrial scale.

4.6.3 Recycling of the hydrolysis residue and sequential hydrolysis

The effect of solids-recycling and sequential hydrolysis were compared to batch hydrolysis of autohydrolysed and delignified straw. Solids-recycling was found to improve volumetric productivity (Figure 22A) by increasing the reaction consistency, without reducing hydrolysis yields (Figure 22B). This resulted from the removal of sugars, thus reducing product inhibition, and the removal of part of the liquid during the process, thereby decreasing the reaction volume. However, equal benefits were achieved by sequential hydrolysis, indicating that no enzyme accumulation beyond the accumulation of the solid residue takes place in solids-recycling. This renders solids-recycling a method of product removal, not enzyme recycling. Enzyme recycling would require accumulation of enzymes by net migration of enzymes from the residue to the fresh substrate. This could be particularly expected from delignified straw, where the solids are almost completely hydrolysed and the enzymes could be assumed to be forced to detach and relocate. In actual fact, the opposite was observed as delignified straw showed even less benefit from the recycling and sequential processes compared to autohydrolysed straw (Figure 22A & B).

The reaction time of the processes was equalized, based on the realization that the average reaction time in solids recycling follows a geometrical series until reaching steady state (Eq. 17). This is an important consideration that has previously been neglected. Weiss *et al.*[169] reported a 33 % reduction in enzyme demand by 85% solids recycling. This was undoubtedly caused by the increase of the reaction time from 3 to 20 days, which was not discussed. Earlier reports have focused on the determination of the total residual bound activity, instead of a continuous process at steady-state.[21,166,168]

$$t = \sum_{i=0}^{\infty} t_0 r^i = \frac{t_0}{1-r} \qquad (17)$$

In this study, a solids-recycling reaction with an average reaction time of 48 h (24 h reaction with 50% recycling) was compared to a sequence of two 24 h reactions (Figure 22C). Additionally, both processes were continued with an additional 24 h hydrolysis step without addition of enzymes. With batch reactions as references, an equal amount of water was applied in each overall process by dividing it between the sequential reactions, thus eliminating dilution. Similar conclusions were drawn from both the 48 and 72 h processes. Equalizing the liquid feed and the reaction time led to an increase in the reaction consistency from 10% to 16% DM and from 20% to 27% DM with both solids recycling and sequential hydrolysis (9 FPU g^{-1}). This allocated the benefit of product removal

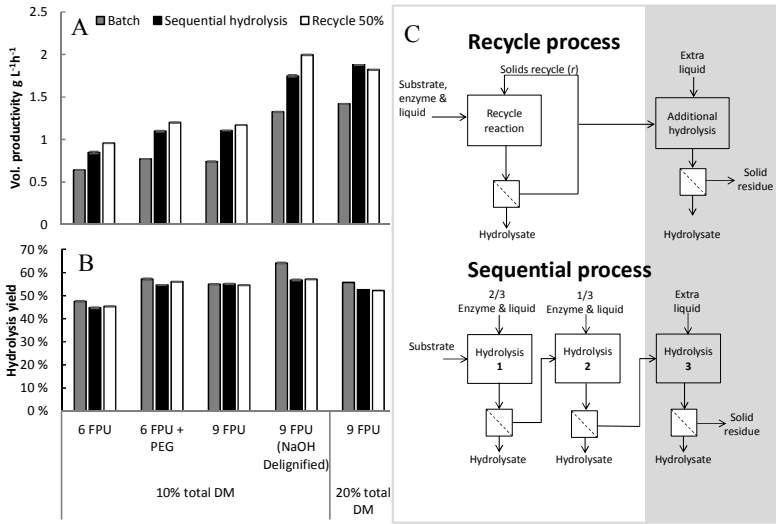

***Figure 22**. A) Volumetric productivity and B) hydrolysis yield of solids-recycling, sequential hydrolysis and batch hydrolysis in a 48 h process for AH-straw, AH-straw with PEG-addition and NaOH-straw. C) The total 72 h process flow of a solids-recycling process (48 h) with an additional 24 h reaction and a sequential hydrolysis process with three 24 h reactions.*

into an increased volumetric productivity, whereas the hydrolysis yields were unchanged in comparison to the corresponding batch reactions at 10% and 20% DM. Since the resulting sugar concentrations were also similar, the similar hydrolysis yields at different consistencies imply that the generally negative effect of consistency[78] on hydrolysis is predominantly a result of increased product concentration rather than increased viscosity or constrained mass transfer. On the other hand, a set of unpublished preliminary reactions (Appendix I) showed an increase in hydrolysis degree by sequential hydrolysis as well as solids-recycling, when additional water was used to replace the separated liquid. The increase was 10% of in average, whereas over 35% increases have been reported, if the sugars are completely removed by washing at each step[165,205] This means that the benefit from product removal is interchangable between volumetric productivity and hydrolysis yield. If the liquid feed is constant, product removal improves volumetric productivity and if the reaction volume is constant, the hydrolysis yield is improved because of dilution by replacement water.

Solids recycling and sequential hydrolysis did not change the effect of the hydrolysis additive polyethylene glycol (unpublished result). A PEG addition of 1% per DM of autohydrolysed straw increased the hydrolysis with 6 FPU g^{-1} to the level of 9 FPU g^{-1}, in accordance with previous reports.[159,160,162]

The benefit from product removal is clear, but it may be difficult to achieve in industrial scale, since filtration of the hydrolysate may be difficult.[206] Filtration of the high-solids hydrolysates of autohydrolysed straw in **V** straw was initially efficient when a filter cloth was used that allowed the smallest particles to flow through, contrary to filter paper (Whatman 1 & 3). However, the filtration was quickly suppressed by cake resistance, which could possibly be addressed by cross-flow filtration.[207] Therefore, further research should be directed to affordable and efficient separation techniques of lignocellulose hydrolysates in order to enable product removal during reaction.

5 Conclusions

Fractionation of wheat straw into sugars was studied from the perspectives of both process feasibility and scientific understanding. By benchmarking the optimized sugar yield from straw fractionation to sugar market value, true potential for profitability was observed. However, the sugar value had a larger effect than the choice of the fractionation process, thus demonstrating that the economics of lignocellulosic sugars are more affected by factors external to process development. Nevertheless, important improvements can be achieved by different process solutions and yield optimization.

Delignification led to a superior hydrolysability and a higher sugar yield potential compared to a hydrothermal reaction, but delignification suffers from the costs of chemical consumption and recycling. Hydrothermal reaction was particularly efficient in revealing cellulose surfaces, while alkaline delignification improved the pore accessibility and rendered the surface chemistry of the residual lignin less harmful for hydrolysis. Chemical modification of lignin surfaces could therefore also be an advantageous approach for hydrothermal pretreatments. Although amorphous cellulose is more efficiently hydrolysed than crystalline, the majority of cellulose remained crystalline in hydrothermal and delignification pretreatments. Therefore, the most important factor improving hydrolysability was the accessibility of cellulose, whereas the presence of lignin was the most important negative factor.

The hydrolysis rate was found to depend strictly on the hydrolysis degree and no time-dependent effects occurred, other than hydrolysis itself, which simplifies the efforts to explain and model hydrolysis kinetics. The surface area of cellulose decreased linearly with the reduction of cellulose content. Lignin surface area was slightly decreased by dissolution of phenolics, and no fresh lignin surfaces were revealed by cellulose hydrolysis. A Michaelis-Menten type kinetic model was used to study the effects of different possible inhibitory factors arising during hydrolysis. It was concluded that the model should include product inhibition, irreversible inhibition and reduction of cellulose hydrolysability, while being aware that they may not correlate linearly with hydrolysis. This provides a guideline for further modeling efforts, as well as an improvement for the previous NREL-model.

There are no magic tricks expected within the current paradigm of lignocellulose fractionation, but some improvements can be achieved by alternative process configurations. Improvements in this study were achieved with flow-through operation in delignification, which was also found to be applicable at industrial

scale, and with product removal during enzymatic hydrolysis. Both of these process solutions are associated with technical challenges, which require further research. In general, lignocellulose fractionation is a robust process and some elaborate considerations were shown to be less effective than expected. These included counter-current flow-through delignification, which did not bring additional benefit compared to direct flow-through, as well as recycling of the solids in hydrolysis, previously misconcieved as a strategy for enzyme recycling, but now redefined merely as a method for product removal. Flow through processes are a promising for improving pretreatment efficiency, and thus call for further studies for large scale operability and for validation of the volumetric productivity determined by scale up simulation in this study. Hydrolysis is improved by removal of the produced sugars during the reaction while exploiting enzyme adsorption on the solids for enzyme retention. However, more research is needed for efficient separation of the residual solids at large scale to allow product removal.

The development of fuels and chemicals from lignocellulose materials is a major frontier in the ongoing transition from fossil resources towards renewable alternatives. Although the recent decrease in oil prices may temporarily constrain the profitability of the new technologies more vulnerable to competition, the increasing availability of renewable fuels ensures an uninterrupted energy supply when fossil oil reserves eventually decline. While economics will ultimately determine the rate of the transition, the incentive against climate change and oil-dependence is strong, and lignocellulosic biofuels and chemicals are waiting for the next wave of pioneers to push the boundaries forward.

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APPENDIX I

Re-calibration of the hydrolysis model (Eq. 8) for autohydrolysed straw, addition of PEG and product removal

As shown in the Figure S1A, the hydrolysability of the AH-straw in **I** was lower compared to the AH straw studied in **III** and **V** (including unpublished hydrolysis results for the same material at 10% DM for 72 h at 50 °C, 200 rpm). On the other hand, the hydrolysability of NaOH-delignified straw in **I** (12% NaOH) was similar to NaOH-delignified material studied in and **III** and **V** (Figure S1B). Therefore, the model for the hydrolysis yield form AH-straw (Eq. 8) was recalibrated to fit the hydrolysability in **III**, **V** and the unpublished results by re-fitting the parameters $Y_{E,max}$, a and b (Table S1).

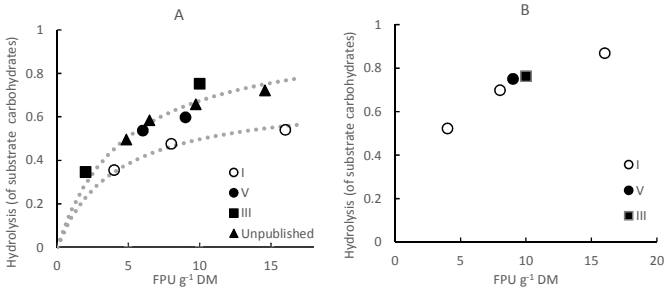

Figure S1. *A) Hydroloysability of autohydrolysed straw in **I** compared to that in **III** and **V**. B) Hydrolysability of delignified straw in **I** compared to that in **III** and **V**.*

The effect of the addition of PEG (1% per DM) was similarly calibrated with the unpublished results of hydrolysis of the same AH-straw that was used in **III** and **V** (Figure S2A).

The effect of product removal with extra water addition was calibrated with unpublished results with a 72 h reaction time (Fig S2B). The data included a set of three sequential hydrolysis reactions, each with three 24 h hydrolysis steps and separation (as in **V**) at three different enzyme dosages. The initial reaction consistency was 10%, but since extra water was added for maintaining a constant reaction volume, the overall process was diluted, corresponding to a consistency

of 5.3%. Additionally, the set included a single result from a 60% solids-recycling reaction and an additional reaction with an overall dilution from the initial 10 to 8.5%.

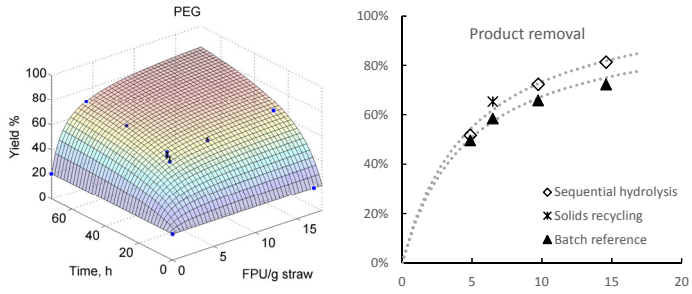

Figure S2. *Recalibration of model for PEG-addition (1% of DM) and product removal.*

Table S1. *Recalibrated parameters for Eq. 8.*

	$Y_{E,max}$	a	b	R^2
AH-straw	80.23	0.244	0.2986	0.979
AH-straw + PEG	84.56	0.058	0.6328	0.987
AH-straw + product removal	88.47	0.244	0.2858	0.996

A major strategy for turning lignocellulose materials into renewable fuels and chemicals relies on enzymatic decomposition of cellulose and hemicellulose into sugars, which are then fermented into biorefinery products. Cellulose, however, is very resistant to enzymatic hydrolysis and the raw materials must therefore be pretreated using high temperatures and chemicals. This thesis compares the sugar yield potential of two major pretreatment categories using wheat straw as raw material, provides a model for yield optimization and elucidates the material properties and inhibition kinetics that affect enzymatic hydrolysis of lignocellulose materials. Improvements to saccharification are presented by using flow-through processes in pretreatment and product removal during hydrolysis by recycling of the hydrolysis residue.

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